Factors influencing the Δ6-desaturation of linoleic acid

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by

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To my mother and father,
with thanks

Declaration

I declare that the work for this thesis was undertaken during my PhD studentship at the Cardiovascular Research Unit, Faculty of Medicine and written up thereafter. I was the principle contributor to all sections except where indicated in the text.

Jonathan E. Brown

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Abbreviations

ACP	Acyl carrier protein
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BB	Bio Breeding
BB/E	Bio Breeding/Edinburgh
BB/O	Bio Breeding/Orléans
BHT	Butylated hydroxy toluene
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CHD	Coronary heart disease
CoA	Coenzyme A
CP	Creatine phosphate
CTP	Cytodine triphosphate
D	Desaturation
DBI	Double bond index
DENS	Data evaluation of non-linear standard curves
E	Elongation
EFA	Essential fatty acid
FAME	Fatty acid methyl ester
GHb	Glycosylated haemoglobin
GLC	Gas liquid chromatography
INT	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-tetrazolium chloride
MUFA	Monounsaturated fatty acid (s)
NAD^+	Nicotinamide adenine dinucleotide (oxidised form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
PC	Phosphatidyl choline
PE	Phosphatidyl ethanolamine
PI	Phosphatidyl inositol
PUFA	Polyunsaturated fatty acid (s)
PMS	Phenazine methosulphate
R_f	Relative flow rate
RNA	Ribonucleic acid
SDH	Succinate dehydrogenase
SFA	Saturated fatty acid (s)
SMR	Standard mortality ratio
TLC	Thin layer chromatography
TMS	Trimethyl silyl
UDP	Uridine diphosphate
WHO	World Health Organisation

Abstract

Δ6-desaturase is located in a pivotal position in the metabolism of essential fatty acids and a low activity may lead to coronary heart disease (CHD). Experimental evidence suggests that Δ6-desaturase is low in humans and data from cross-cultural and case-control studies using fatty acid compositional analyses propose that its activity is reduced by risk markers for CHD. In this thesis the role of some factors implicated in CHD such as diabetes, hypercholesterolaemia and stress are considered and their effect on Δ6-desaturase activity and tissue fatty acid composition is assessed in the rat.

The assay for the Δ6-desaturation of linoleic acid was re-examined and optimised. Microsomal protein and albumin concentrations were found to be critical because of their substrate binding effects. It was also found necessary to measure the amount of non-esterified linoleic acid in microsomal preparations as it competes with the radioactive substrate.

The role of dietary cholesterol on both linoleic acid Δ6-desaturation and tissue fatty acid composition was assessed. Cholesterol enrichment increased Δ6-desaturase activity and this was reflected in the increased product-precursor ratio of γ-linolenic to linoleic acid in microsomal phospholipids. However, the total amount of long chain polyunsaturated fatty acids derived from linoleic acid, including arachidonic acid, was reduced in cholesterol fed rats, suggesting an impaired Δ5-desaturase. The increased activity of liver microsomal Δ6-desaturase was not associated with any significant changes to adipose tissue n-6 fatty acid composition.

Further studies were conducted to elucidate possible reasons for the disparity between the present and published studies on the effect of cholesterol. Cholesterol feeding was carried out in grouped and isolated rats and microsomal Δ6-desaturase reassessed. Although stress caused by isolation reduced Δ6-desaturase activity, dietary cholesterol increased its activity again. This was observed under both saturating and non-saturating substrate conditions with a good correlation observed between the two. Levels of non-esterified fatty acids in the microsomal preparations were not different

between the four groups, indicating that this was not the explanation for the lower activity of Δ6-desaturase reported by others.

The effect of dietary cholesterol on the *in vivo* metabolism of linoleic acid was subsequently pursued to investigate whether this differed from that demonstrated *in vitro*. The overall metabolism of linoleic acid, assessed on the basis of the decline in the specific activity of linoleic acid with time, was decreased by cholesterol feeding, whilst Δ6-desaturase activity of isolated liver microsomes increased in one experiment and no change was found in the remaining experiments.

Further studies examined linoleic acid metabolism in the spontaneous diabetic BB/E rat. Using the optimised Δ6-desaturase assay system, the activity of Δ6-desaturase was decreased by 25 % in diabetic rats treated with insulin compared with controls. Withdrawal of insulin for 54 h reduced microsomal Δ6-desaturase activity slightly further. Microsomes from BB/E diabetic rats in whom insulin was withdrawn contained high levels of non-esterified linoleic acid, which made it necessary to correct for the specific activity of [1-^{14}C]linoleic acid in the estimation of Δ6-desaturase activity. Δ6-desaturase activity as indicated by microsomal phospholipid fatty acid composition, varied depending on which ratio was used. Adipose tissue fatty acid composition was not altered in a manner consistent with a depressed Δ6-desaturase activity in diabetic rats.

These results show that there is no consistent effect on Δ6-desaturase by risk factors for CHD such as raised cholesterol, diabetes and 'stress' and that tissue fatty acid compositions do not adequately relate to changes in the activity of Δ6-desaturase in liver microsomes.

Contents

Factors influencing the Δ6-desaturation of linoleic acid

Chapter 1 Literature review

Chapter 2 Materials and methodology

Chapter 7 Linoleic acid metabolism in the BB/E diabetic rat

Chapter 1

Literature review

1.1 Introduction

Lipids constitute a major component of Western diets (Fehilly, 1984). They make food more palatable but also provide a good source of energy and nutrients such as the fat soluble vitamins and essential fatty acids. Yet about 100 years ago dietary fats were perceived as insignificant (Lawes and Gilbert, 1877) and more recently it was believed that the beneficial effects of fat could be ascribed to the lipid soluble vitamins or to the caloric effects of fat itself (McAmis *et al.*, 1929). Rigorous dietary fat exclusion experiments by Burr and Burr eventually provided evidence for the essentiality of fat (Burr and Burr, 1929; Burr and Burr, 1930). They described how an acute deficiency state, characterised by impaired fertility, low growth rates and dermatitis, could be produced in Long-Evans rats maintained on a fat-free diet and concluded that linoleic acid was an essential fatty acid (EFA).

Many years later, EFA deficiency in humans was described (Collins *et al.*, 1971; Wene *et al.*, 1975) in patients receiving long-term naso-gastric and intravenous feeds. A similar skin condition to that manifest in rats has been shown in infants receiving formula feeds deficient in EFA (Holman *et al.*, 1964). Other reported defects arising from human EFA deficiency include increased transepidermal water loss, modified pulmonary and digestive function and altered liver morphology (Yamanaka *et al.*, 1982).

Full blown EFA deficiencies rarely exist but the role of a moderate deficiency in disease processes has been speculated upon. Sinclair (1956) suggested that changes in food manufacturing processes, especially those concerned with refining food oils to improve shelf-life (first introduced by Sabatier and Senderens in 1897), could make diets *moderately* EFA deficient. He posed the question, do diets 'high in saturated or unnatural fats' and 'low in EFA' exist? And speculated that some of the chronic

diseases characteristic of Western 'civilised' society, such as atherosclerosis and cancer, resulted from this dietary manipulation. Many studies have since been devised to assess this hypothesis but prior to examining the strengths and weaknesses of such studies it is necessary to go briefly through properties of EFA and characteristics of the enzymes responsible for EFA metabolism.

1.2 Structure, occurrence and nomenclature of EFA

Two series of EFA exist. They are termed n-6 and n-3 and can be differentiated by the location of the first double bond from the methyl end of the molecule. They contain two or more *cis* double bonds in a straight chain of between 18 and 22 carbon atoms in length. Linoleic and α-linolenic acids are the *parent* fatty acids of the n-6 and n-3 series, respectively. Other important members can be derived from these acids in humans by an alternating sequence of oxidative desaturation and elongation.

The essentiality of the so called *parent* EFA is due to the inherent inability of mammals, including humans, to desaturate oleic (*cis*, 9-octadecenoic) acid between the centrally located double bond and the methyl terminus. Only terrestrial plants (Gurr *et al.*, 1969; Stymne and Appelqvist, 1978; Stymne *et al.*, 1992), marine phytoplankton and some protozoa and insects (Borgeson *et al.*, 1990) have the cellular machinery for this specific task. Within such organisms Δ12-desaturase inserts a *cis* double bond (between carbon atoms 12 and 13) into oleic acid to form linoleic (*cis*, *cis*, 9, 12-octadecadienoic) acid (Fig. 1.1).

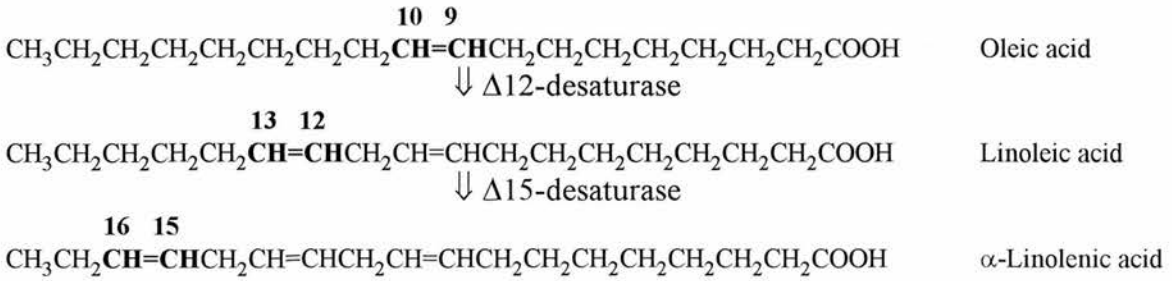


Figure 1.1 *Essential fatty acid synthetic scheme in plants (bold numbers indicate site of cis double bond insertion numbering from the carboxylic end)*

In higher plants, a further site-specific modification by Δ15-desaturase yields α-linolenic (*cis*, *cis*, *cis*, 9, 12, 15-octadecatrienoic) acid (Fig 1.1). Therefore in plants the parent n-6 and n-3 fatty acids are interconvertible (n-6 formed from n-3) but as humans and mammals do not possess Δ15-desaturase these EFA are entirely separate.

Membrane and storage lipids in both animals and plants form the predominant intake of EFA in humans. Animal fats, such as butter, contain a preponderance of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) with only minor amounts of n-6 and n-3 polyunsaturated fatty acids (PUFA) (2.2 and 1.0 %, respectively (Table 1.1)). Natural seed oils such as olive oil contain approximately 80 % oleic and 7 % linoleic acids whereas sunflower and corn oils contain a high level of linoleic acid (52 and 75 % by weight, respectively).

Table 1.1 *Proportions of principle fatty acids in selected animal, marine and vegetable sources*

	Fatty acid [a]							
Oil/fat source	Palmitic	Stearic	Oleic	Linoleic	γ-LA #	α-LA	EPA	DHA
Animal								
Butter	28	12	30	2	-	1	-	-
Beef tallow	27	7	48	2	-	1	-	-
Marine								
Menhaden	21	3	11	1	-	3	13	8
Plant								
Olive	10	2	78	7	-	1	-	-
Sunflower	7	3	14	75	-	<1	-	-
Evening primrose	5	2	10	74	9	<1	-	-
Corn	13	3	31	52	-	1	-	-
Linseed	6	3	17	14	-	60	-	-

Values expressed as % (w/w) total fatty acids. [a] Data compiled from Gunstone *et al.* (1986) and Abraham *et al.* (1990). # γ-LA=γ-linolenic acid , α-LA=α-linolenic acid, EPA=eicosapentaenoic acid, DHA=docosahexaenoic.

A simple abbreviated notation is often used to express the structures of fatty acids and acyl groups (Table 1.2). Linoleic and α-linolenic acids are written 18:2 n-6 and 18:3 n-3, respectively. The first number in the notation refers to the number of carbon atoms in a straight chain and the digit following the colon refers to the number of methylene interupted *cis* double bonds. Small n represents the total number of carbon atoms in the chain and n-6 (n minus 6) refers to the number of carbon atoms from the methyl end of the molecule to the first double bond. This notation will be used frequently throughout this thesis.

Table 1.2 *Common fatty acids and their nomenclature*

Common name	Systematic name	Abbreviated notation
SFA		
Palmitic	hexadecanoic	16:0
Stearic	octadecanoic	18:0
Arachidic	eicosanoic	20:0
MUFA		
Palmitoleic	*cis*, 9-hexadecenoic	16:1 n-7
Oleic	*cis*, 9-octadecenoic	18:1 n-9
Vaccenic	*cis*, 11-octadecenoic	18:1 n-11
Gadoleic	*cis*, 11-eicosaenoic	20:1 n-9
PUFA (n-6)		
Linoleic	*cis*, *cis*, 9, 12-octadecadienoic	18:2 n-6
γ-Linolenic	all *cis*, 6, 9, 12-octadecatrienoic	18:3 n-6
Dihomo-γ-linolenic	all *cis*, 8, 11, 14-eicosatrienoic	20:3 n-6
Arachidonic	all *cis*, 5, 8, 11, 14-eicosatetraenoic	20:4 n-6
Adrenic	all *cis*, 7, 10, 13, 16-docosatetraenoic	22:4 n-6
Docosapentaenoic	all *cis*, 4, 7, 10, 13, 16-docosapentaenoic	22:5 n-6
PUFA (n-3)		
α-Linolenic	all *cis*, 9, 12, 15-octadecatrienoic	18:3 n-3
Stearidonic	all *cis*, 6, 9, 12, 15-octadecatetraenoic	18:4 n-3
Eicosatetraenoic	all *cis*, 8, 11, 14, 17-eicosatetraenoic	20:4 n-3
Eicosapentaenoic	all *cis*, 5, 8, 11, 14, 17-eicosapentaenoic	20:5 n-3
Clupanodonic	all *cis*, 7, 10, 13, 16, 19-docosapentaenoic	22:5 n-3
Docosahexaenoic	all *cis*, 4, 7, 10, 13, 16, 19-docosahexaenoic	22:6 n-3
PUFA (n-9)		
Mead	all *cis*, 11, 14, 17-eicosatrienoic	20:3 n-9

Numbers within systematic nomenclature e.g. *cis, cis,* 9,12-octadecadienoic refer to the location of the double bonds in the carbon chain counting from the carboxylic end of the molecule.

In addition to the small number of n-6 and n-3 fatty acids present in nature, several non-essential fatty acids exist. Most are termed either saturated or monounsaturated (Table 1.2) and can be derived either from the diet or *de novo* synthesis. Desaturation

of SFA by Δ9-desaturase yields the respective monoene. This elimination of two *cis* H-atoms from SFA such as 18:0 reduces the melting point and is believed to facilitate biochemical processing of entities such as lipoproteins (Jeffcoat, 1979).

1.3 Outline of EFA metabolism

EFA have an indispensable function as integral components of membrane phospholipids involved in the regulation of cellular functions (Stubbs and Smith, 1984; Brenner, 1984; Spector and Yorek, 1985). They also have an important role in the production of prostanoids in various physiological and pathological processes. Linoleic acid is an important structural component of skin (Melton *et al.*, 1987; Hansen and Jensen, 1985) and also a regulator of cholesterol transport (Horrobin and Manku, 1983). Yet in order to perform its *full* physiological and structural roles it must be converted to more unsaturated, longer chain n-6 fatty acids. This is achieved by an alternating sequence of desaturation and elongation steps with the same pathway being used for both 18:2 n-6 and 18:3 n-3 (Fig. 1.2). Oleic acid can also be converted in the same way to 20:3 n-9 but this fatty acid, which accumulates in EFA deficiency, cannot entirely replace n-3 or n-6 long chain fatty acids.

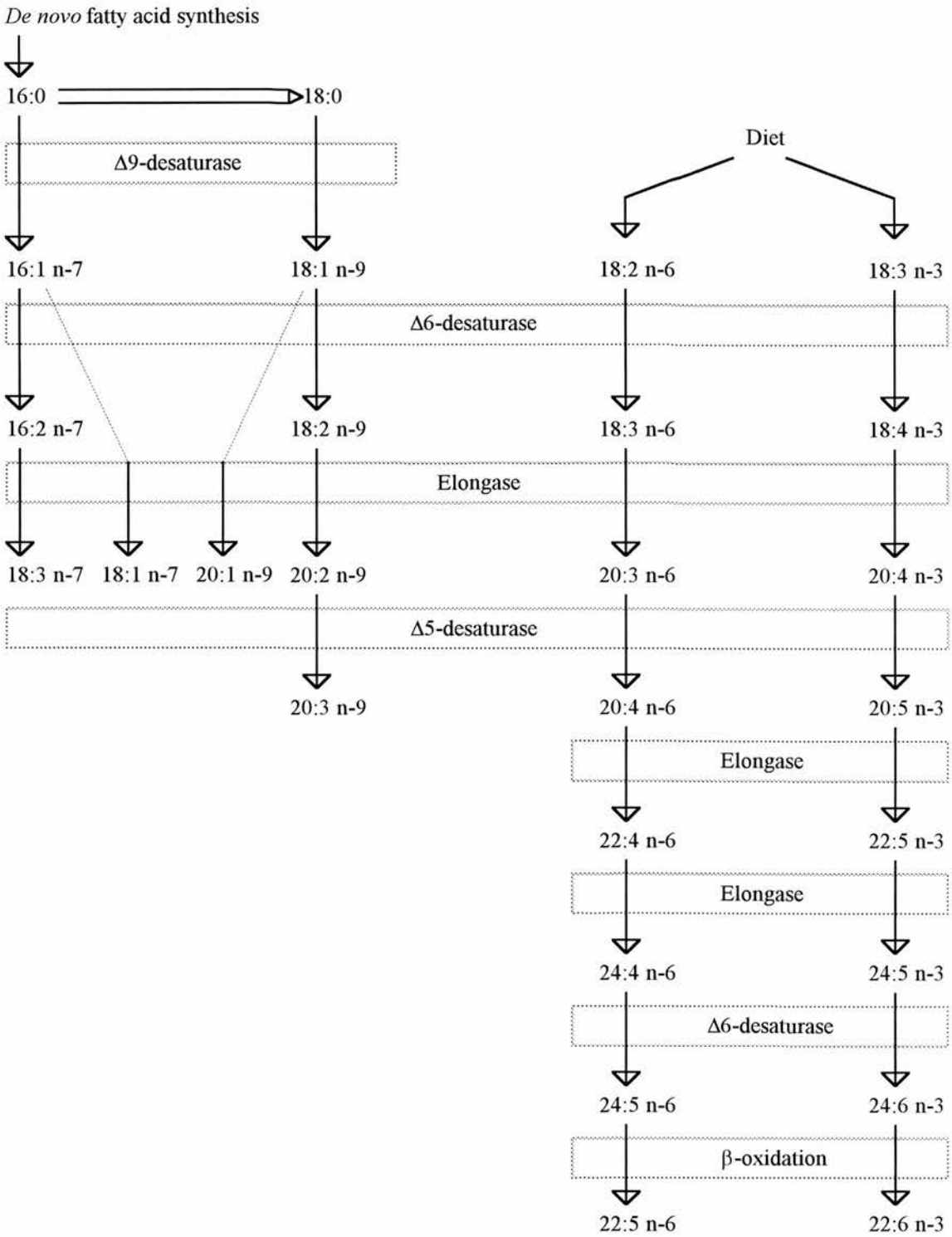


Figure 1.2 *Mammalian fatty acid conversions. All fatty acids are in acyl-CoA form.*

As for many metabolic inter-conversions that involve fatty acids, appropriate activation by esterification with coenzyme A is necessary for further metabolism (Groot *et al.*, 1976). The endoplasmic reticulum, site for desaturation and elongation, contains long chain fatty acyl-CoA synthetases [EC 6.2.1.3] that utilise ATP, CoA and Mg^{2+} to form acyl-CoA derivatives (Kornberg and Pricer, 1953; Groot *et al.*, 1976; Normann *et al.*, 1981). This two-step process, with an intermediary compound fatty acyl-AMP, also takes place in mitochondria (De Jong and Hülsmann, 1970; Aas, 1971) and peroxisomes (Waku, 1992). The activity of acyl-CoA synthetase in liver microsomes is high (50-100 nmol/min/mg protein; Aas, 1971; Normann *et al.*, 1981) and not considered rate limiting in the metabolism of EFA. Further characteristics of this enzyme have been reviewed in some detail (Groot *et al.*, 1976) and will not be discussed here.

Once activated, linoleoyl-CoA undergoes stereo-specific abstraction of two H-atoms to form a *cis* double bond. The process is carried out by Δ6-desaturase, so termed because it abstracts H-atoms from carbons 6 and 7 (numbering from the carboxyl end of the fatty acid). The resultant product contains three methylene ($-CH_2-$) interrupted *cis* double bonds and is termed γ-linolenyl-CoA. Further metabolism by the microsomal elongase adds two carbon atoms provided by malonyl-CoA (in contrast to malonyl-ACP used by fatty acid synthetase) and forms dihomo-γ-linolenyl-CoA. Oxidative desaturation of dihomo-γ-linolenyl-CoA by Δ5-desaturase (stereo specific H-atom abstraction from carbons 5 and 6) yields arachidonyl-CoA (Fig. 1.2).

Up until very recently it was assumed that Δ4-desaturation ensued the elongation of 20:4-CoA to adrenyl-CoA. This is now known not to be the case. The revised pathway is now considered to consist of three separate steps (Voss *et al.*, 1991, Sprecher, 1992). First, adrenyl-CoA undergoes elongation to 24:4 n-6 CoA; second, oxidative Δ6-desaturation of 24:4 n-6 CoA forms 24:5 n-6 CoA; and finally, partial peroxisomal β-oxidation of 24:5 n-6 CoA yields 22:5 n-6 CoA. The equivalent pathway also proceeds for n-3 fatty acids (Voss *et al.*, 1991). Therefore in the standard

mammalian system, Δ6-desaturase is placed in a unique and pivotal location as six fatty acids (one n-7, one n-9, two n-6 and two n-3 acids) can all compete at the level of this desaturase.

Rates of both desaturation and elongation have been measured extensively in liver microsomes. The desaturation step is relatively slow and this is particularly true of Δ6-desaturase (25-600 pmol/min/mg). Fatty acid elongation is approximately five times more rapid than desaturation in rat liver microsomes (Bernert and Sprecher, 1975). As a consequence, levels of fatty acids in microsomal lipids often show a pattern that reflects rapid elongation and slow desaturation.

Dietary, hormonal and pharmacological modulators, as well some related disease states, have received considerable attention in connection with alterations in the metabolism of EFA. The position of Δ6-desaturase in EFA metabolism means that it plays a pivotal role in the formation of long chain PUFA. Prior to discussing some of these modulators in detail it is essential to outline the enzymology of Δ6-desaturase.

1.4 Enzymology of Δ6-desaturation

In mammalian and avian systems, the oxidative desaturation of fatty acyl-CoA is accomplished by a terminal desaturase moiety coupled to a mini-electron transport chain associated with the endoplasmic reticulum. Much of the preliminary information on such systems has been established with stearoyl-CoA desaturase (Bernhard *et al.*, 1959; Marsh and James, 1962). This complex contains a flavoprotein, NADH-cytochrome b_5 reductase (Holloway and Wakil, 1970) and a haem-protein, cytochrome b_5 (Oshino *et al.*, 1966; Oshino *et al.*, 1971; Holloway and Katz, 1972; Shimakata *et al.*, 1972) as components of the mini-electron transport chain. Data supporting the involvement of these two electron transport proteins in linoleoyl-CoA desaturation has also been demonstrated (Lee *et al.*, 1977; Okayasu *et al.*, 1977; Okayasu *et al.*, 1981). Both desaturase complexes have a requirement for molecular oxygen and NADH (or NADPH) and are sensitive to cyanide but not carbon monoxide (Okayasu *et al.*, 1981).

In the linoleoyl-CoA desaturase complex, the terminal moiety is Δ6-desaturase while for stearoyl-CoA desaturase the terminal component is Δ9-desaturase.

In 1974 Strittmatter and co-workers confirmed the presence of the three components in stearoyl-CoA desaturase by purifying the complete desaturase system from rat liver (Strittmatter *et al.*, 1974). Prasad and Joshi (1979) reproduced these findings in chicken liver. Linoleoyl-CoA desaturase has also been purified (Okayasu *et al.*, 1981). The schematic layout of this complex is shown in Fig. 1.3.

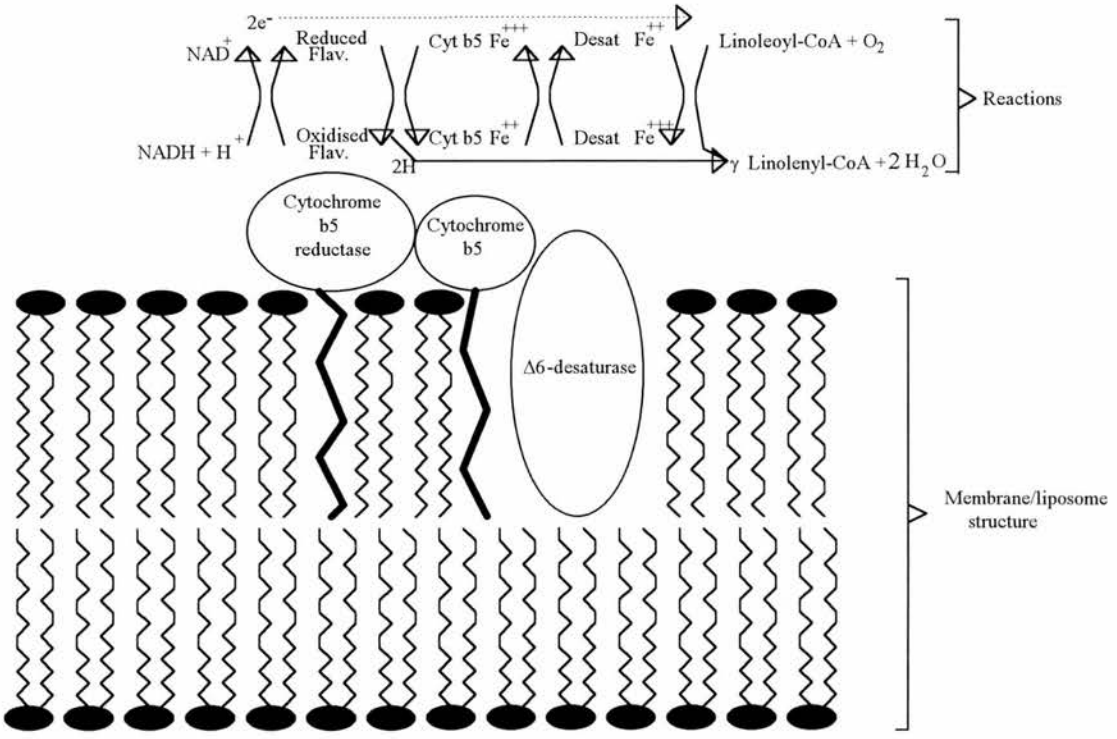


Figure 1.3 *Hypothetical structure of the linoleoyl-CoA desaturation system within the lipid bilayer*

1.4.1 NADH-cytochrome b_5 reductase

NADH-cytochrome b_5 reductase [EC 1.6.2.2] was originally isolated from microsomes using cobra venom (Strittmatter and Velick, 1957) but subsequent isolations have employed lysosomes (Takesue and Omura, 1970) and detergents (Spatz

and Strittmatter, 1973). This flavoprotein has a molecular weight 44,000 (Spatz and Strittmatter, 1973) and contains distinct hydrophilic (MW 33,000) and hydrophobic domains (MW 11,000). The hydrophilic domain, which is projected into the surrounding cytosol, contains the active site FAD. The hydrophobic domain anchors the protein by strong non-covalent interactions with the lipid bilayer.

1.4.2 Cytochrome b_5

Cytochrome b_5 exists in large amounts in liver cell endoplasmic reticulum and has been isolated using either proteolytic enzymes (Omura *et al.*, 1967) or detergents (Ito and Sato, 1968). It contains 133 amino acids and has a molecular weight of 16,700. It is amphipathic (Tajimura *et al.*, 1978) and projects the hydrophilic domain containing 97 amino acid residues into the cytosol. The hydrophobic chain of 44 amino acids anchors the bulk of the protein to the membrane (Sullivan *et al.*, 1973; Enomoto and Sato, 1973).

1.4.3 Terminal Δ6-desaturase

The terminal moiety of linoleoyl-CoA desaturase was first isolated by Okayasu and co-workers in 1980 (Okayasu *et al.*, 1981). It was partially characterised at this time and found to contain one atom of non-haem iron per molecule of enzyme. Further analysis revealed that the enzyme was comprised of a single polypeptide chain with a molecular weight of approximately 66,000. Reconstitution of the terminal Δ6-desaturase with NADH-cytochrome b_5 reductase, cytochrome b_5 and lipid (or detergent) demonstrated the ability to desaturate 18:2-CoA to 18:3-CoA in the presence of NADH and molecular oxygen (Okayasu *et al.*, 1981). The desaturase was inactive toward 18:0-CoA but substrates such as α-linolenyl-CoA and oleoyl-CoA were unfortunately not examined.

The linoleoyl-CoA desaturase terminal moiety shows some similarities with Δ9-desaturase (Table 1.3). This latter protein is probably the most studied and well

understood desaturase in both biochemical and molecular terms. It is smaller than Δ6-desaturase but contains a slightly higher proportion of non-polar amino acid residues.

Table 1.3 *Comparison of mammalian Δ6- and Δ9-desaturase characteristics*

Measurement	Δ6-desaturase [a]	Δ9-desaturase [b]
Molecular weight	66,000	53,000
Total amino acid residues	598	458
Non-polar residues (%)	49	62
Inhibition by iron chelators	Yes	Yes
Inhibition by *p*CMBS	Yes	Yes
Inhibition by cyanide	Yes	Yes
Gene isolation	No	Yes
Gene expression	No	Yes

[a] Data compiled from Okayasu *et al.* (1981). [b] Data compiled from Strittmatter *et al.* (1974), Thiede *et al.* (1986) and Strittmatter *et al.* (1988). *p*CMBS, *p*-chloromercuribenzene sulphonate. Pugh and Kates (1979) have partially purified Δ5-desaturase. However, no studies have been reported on the purification of the complex or the terminal desaturase to homogeneity.

Both reconstituted desaturases are inhibited by iron chelators, cyanide and *p*-chloromercuribenzene sulphonate. However, studies with the Δ9-desaturase have gone further establishing the rate limiting step in desaturation as hydrogen abstraction (Enoch *et al.*, 1976). Moreover, much of the molecular biology of Δ9-desaturase has been elucidated (Thiede and Strittmatter, 1985; Thiede *et al.*, 1986; Strittmatter *et al.*, 1988; Mihara, 1990). In contrast, since Okayasu *et al.* (1981) purified Δ6-desaturase from rat liver, little progress has been made with either biochemical or molecular biological characterisation of this desaturase in mammalian systems. One of the reasons for this will become evident below (Section 1.4.5).

1.4.4 Cytosolic factors

Accessory factors required for the full activity of Δ6-desaturase were first reported by Brenner (1974) and later by Catalá *et al.* (1975). They showed that the extraction of microsomes, using low ionic strength solutions with subsequent centrifugation, yielded a soluble component that activated the desaturation of 18:2 n-6 in 'washed' microsomes. This lightly bound moiety has no desaturation activity itself (Catalá *et al.*, 1975) and has a lipoprotein-like nature with a protein-lipid ratio of 1.22 (Leikin and Brenner, 1986). The activation of Δ6-desaturase is believed to result from the cytosolic factor binding to the desaturation product, 18:3 n-6 (Leikin and Brenner, 1986). This prevention of retro-inhibition has also been described for Δ5-desaturase (Leikin and Brenner, 1989b). However, it appears that two separate subfractions of the cytosolic fraction prevent Δ5- and Δ6-desaturase retroinhibition independently, by binding to their respective desaturation products (Leikin and Brenner, 1989b).

1.4.5 Desaturation assays

At present a number of different methods are available for the measurement of the oxidative desaturation of SFA and PUFA. In the most commonly used assessments, applicable to all liver desaturases, the relevant radioactive fatty acid or fatty acyl-CoA is employed. For example microsomal Δ6-desaturase, first measured by Nugteren (1962), can be determined using either [1-^{14}C]linoleoyl-CoA (Jeffcoat *et al.*, 1976) or [1-^{14}C]18:2 n-6 (Brenner, 1971) with the appropriate cofactors. The latter, more commonly used substrate, requires activation to the CoA form by acyl-CoA synthetase [EC 6.2.1.3] but this is not considered rate limiting (Brenner, 1971). After an appropriate incubation time (5-20 min), the reaction mixture undergoes alkaline hydrolysis and the resultant non-esterified fatty acids are extracted, methylated and separated by thin-layer (TLC) (Morris, 1966; Christie, 1982; Gardiner and Duncan, 1991), gas-liquid (Brenner *et al.*, 1965; De Schrijver and Privett, 1982) or high pressure liquid chromatography (Narce *et al.*, 1988a).

The original method, employing argentation TLC and liquid scintillation counting, provides the greatest sensitivity as relatively large amounts of lipid (~1 mg) can be separated and counted. This is particularly important in the measurement of Δ6-desaturase as the resultant product, 18:3 n-6, contains a low level of counts. Gas-liquid (GLC) and high pressure liquid chromatography (HPLC) separate small amounts of lipid and therefore product counts can be extremely small. Advantages of GLC and HPLC include their ability to separate 18:3 n-6 and 20:3 n-6. However, this quality of separation is not strictly necessary in the measurement of Δ6-desaturase.

Specifically labelled [9,10-^{3}H]stearoyl-CoA and [9,10-^{3}H]18:0 have been used to assess the activity of Δ9-desaturase (Talamo and Bloch, 1969; Johnson and Gurr, 1971). In contrast to the time consuming methods involving [1-^{14}C]labelled fatty acids the method is relatively quick and simple. Measurement is dependent on ^{3}H release from positions 9 and 10 of 18:0 which is liberated into the aqueous phase as $^{3}H_2O$. Subsequent trichloroacetic acid precipitation of microsomal protein and associated radioactive lipid in the incubation mixture allow $^{3}H_2O$ quantification which is proportional to desaturation. The isotopic effect interferes with the measurement but this is overcome by standardising the assay with [1-^{14}C]18:0 (Johnson and Gurr, 1971). The lack of commercial availability of [6,7-^{3}H]linoleoyl-CoA or [6,7-^{3}H]18:2 n-6 restricts the assay application to Δ9-desaturase.

The microsomal mini-electron transport chain, associated with the oxidative desaturation of fatty acids, has been used as a further method to estimate desaturase activity (Oshino *et al.*, 1971). This spectrophotometric method uses the rate of reoxidation of NADH-reduced cytochrome b_5 in the presence and absence of linoleoyl-CoA as a measure of Δ6-desaturation (Fig. 1.4). Briefly, NADH is added to a dilute microsomal suspension and the time of onset of cytochrome b_5 reoxidation (assessed simultaneously at 424 and 409 nm) is determined in the absence of linoleoyl-CoA (Fig. 1.4, Curve A). In another incubation, NADH and linoleoyl-CoA are added together to the incubation mixture and the time for cytochrome b_5 reoxidation is measured for a

second time (Fig. 1.4, Curve B). The difference in the time due to linoleoyl-CoA induced NADH oxidation (indicated by the difference in cytochrome b_5 reoxidation), is used to calculate the desaturase activity assuming that one mole of NADH is required for the formation of one mole of γ-linolenyl-CoA.

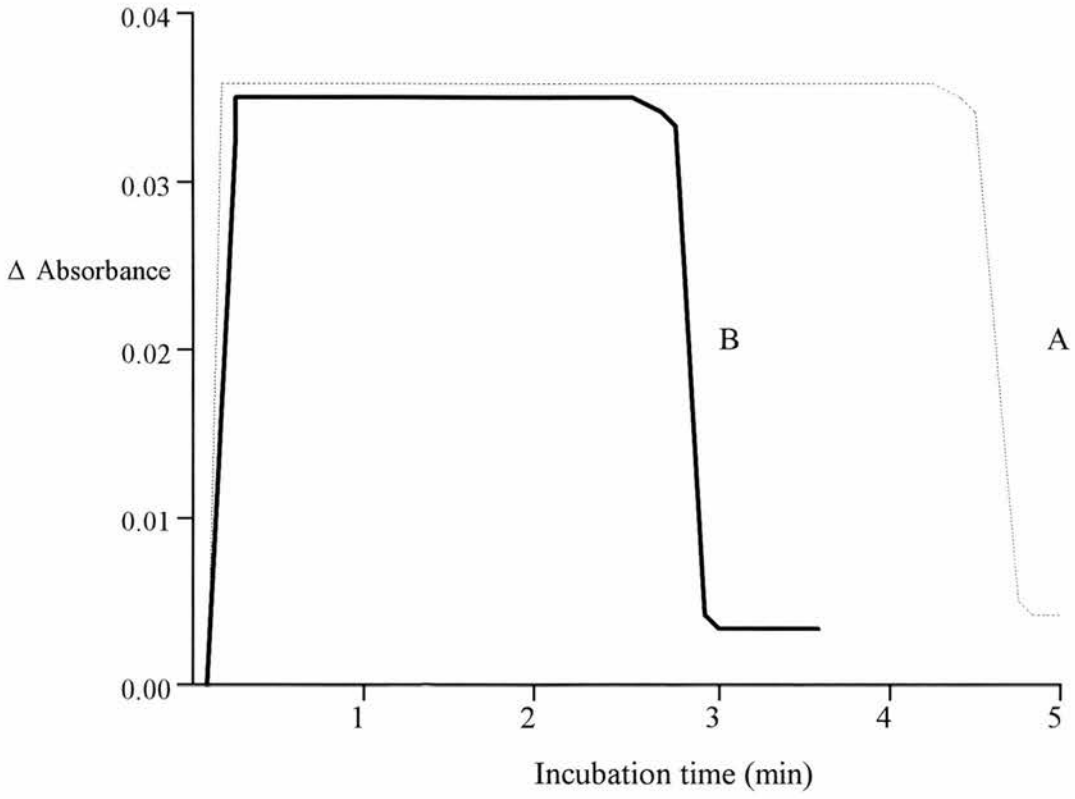


Figure 1.4 *Representation of a cytochrome b_5 reduction and reoxidation trace*

The assay employing cytochrome b_5 reoxidation requires specialised equipment including a dual wavelength spectrophotometer. This type of equipment is expensive and not generally available. As a consequence the assay is not frequently used except when large numbers of samples have to be assayed quickly as in the case of desaturase protein purification.

The metabolism of 18:2 n-6 has also been evaluated using stable isotopes (Emken *et al.*, 1993). Advantages of this method include its applicability to human studies *in*

vivo. However, only a limited number of studies have used stable isotopes owing to the considerable expense of both the substrate, [1-^{13}C]18:2 n-6, and the associated GLC-mass spectrometry analysis equipment.

An indirect measurement of the level of desaturase activity is frequently derived from tissue fatty acid compositional data. In animal studies, for example, product/precursor ratios from liver microsomal phospholipids are generally considered to reflect desaturase activities. This is due to the close proximity of these lipids to the site of desaturation. Ratios typically used for Δ9-, Δ6-, and Δ5-desaturases are shown in Table 1.4.

Table 1.4 *Typical desaturase fatty acid ratios*

Desaturase	Fatty acid ratio
Δ9	18:1 n-9/18:0
Δ6	18:3 n-6/18:2 n-6
Δ6	18:4 n-3/18:3 n-3
Δ6	22:5 n-6/22:4 n-6
Δ6	22:6 n-3/22:5 n-3
Δ5	20:4 n-6/20:3 n-6
Δ6 and Δ5	20:4 n-6/18:2 n-6
Combined D & E of 18:2 n-6	(18:3 n-6+20:2 n-6+20:3 n-6+20:4 n-6+22:4 n-6+22:5 n-6)/18:2 n-6

D desaturation, E elongation.

In human studies the liver is essentially unavailable and as a result plasma and/or adipose tissue fatty acids are frequently used. Yet, criticism can be directed at the use of indices in general as tissue fatty acid composition is not solely dependent on the availability of products derived from desaturation and elongation. Other factors that determine the tissue fatty acid profile include acyl transferase activity, competition for

acyl sites, phospholipase activity, extent of lipid peroxidative processes and requirements for energy and prostaglandins.

Advances in the genetics of Δ6-desaturase in *Synechocystis* (Reddy *et al.*, 1993) will, if homology exists with mammals, provide further means to improve studies in the measurement of the capacity of mammalian tissues to metabolise EFA.

1.4.6 Δ6-desaturase in animals

Nutritional and hormonal studies have frequently focused upon the Δ6-desaturative capacity of liver mainly because it exhibits the highest activity (Ackman and Cunnane, 1992). The animal model most often used is the rat generally because it is easy to study and will consume a wide variety of diets. Other species, however, also possess to differing extents, the capacity to desaturate 18:2 n-6. The mouse (Bourre *et al.*, 1990), pigeon (Borlakoglu *et al.*, 1990), sheep (Shand *et al.*, 1978), dog (Girón *et al.*, 1989) and pig (Clandinin *et al.*, 1985) have all been demonstrated to desaturate 18:2 n-6 (Table 1.5).

Table 1.5 *Hepatic Δ6-desaturase activity in animals §*

Species	Δ6-desaturase activity #
Mouse [a]	40-80
Rat [b]	100-600
Pigeon [c]	310
Cat [d]	n.d.
Cat (EFA-deficient) [e]	detectable using stable isotopes
Dog [f]	~100

§ None of these studies attempted to compare species under controlled dietary conditions. # 18:2 n-6 employed as substrate, pmoles/min/mg microsomal protein. n.d. not detectable, a Bourre *et al.* (1990); b Garg *et al.* (1988a), Christiansen *et al.* (1991); c Borlakoglu *et al.* (1990); d Rivers *et al.* (1976); e Pawlosky and Salem, (1992); f Girón *et al.* (1989).

Once it was believed that felinae could not desaturate either 18:2 n-6 or 20:3 n-6 (Rivers *et al.*, 1976). This is not so as cats do possess Δ6-desaturase during EFA deficiency (Pawlosky and Salem, 1992). It is therefore probable that the rat and cat form two ends of a broad spectrum of desaturation efficiency (Table 1.5).

Some comparisons have been made between Δ6-desaturase activities and the propensity for particular species to develop atherosclerosis. Different animals, however, consume considerably different diets and therefore inter-species comparisons are not valid. The values in Table 1.5 provide only a very rough guideline.

1.5 Dietary modification of Δ6-desaturase activity

Studies examining the effects of dietary modification on Δ6-desaturase activity have generally focused on rat liver microsomes. In such studies different types of lipid, protein, carbohydrate, metal ions and vitamins have been examined. The sections below summarise the findings of some of these studies.

1.5.1 Dietary lipids and Δ6-desaturation

It has been long known that the absence of adequate dietary levels of 18:2 n-6 leads to an elevated Δ6-desaturase activity (Brenner, 1981). Subsequently, the activity of Δ6-desaturase has been measured extensively in dietary manipulation studies using purified fats and oils rich in specific types of lipid (i.e. SFA, MUFA, n-6 PUFA, n-3 PUFA *etc.*). Beef tallow provides an abundance of SFA and MUFA and the feeding of such fat (in a well balanced diet) leads to an increased liver Δ6-desaturase activity compared to a sunflower oil enriched diet (Garg *et al.*, 1990). Similarly, diets high in *trans* fatty acids (Shimp *et al.*, 1982; Kirstein *et al.*, 1983), linseed oil (Garg *et al.*, 1990; Christiansen *et al.*, 1991) and fish oil (De Schrijver and Privett, 1982, Garg *et al.*, 1990, Christiansen *et al.*, 1991) all decrease liver microsomal Δ6-desaturase activity. Dietary oils rich in MUFA (e.g. olive oil) also decrease Δ6-desaturase activity

when compared to rats fed sunflower oil (Mahfouz *et al.*, 1984) as does the incorporation of partially hydrogenated herring oil into rat diets (Svensson, 1983).

Many of the findings *in vivo* have been supported by *in vitro* studies using pure fatty acid preparations. Thus, *trans* 18:1 (Mahfouz *et al.*, 1980) inhibits the conversion of 18:2 n-6 to 18:3 n-6. Oleic acid and 18:3 n-3 also compete with 18:2 n-6 for Δ6-desaturation (Brenner and Peluffo, 1966). The order of affinity of these fatty acids for Δ6-desaturase is 18:3 n-3 > 18:2 n-6 >18:1 n-9. SFA, however, do not affect Δ6-desaturase activity *in vitro* (Brenner and Peluffo, 1966).

Dietary sterols, including cholesterol, also influence EFA metabolism. Some of the first studies on dietary cholesterol enrichment showed an exacerbation of EFA deficiency in rats (Holman and Peifer, 1960). Unfortunately no Δ6-desaturase measurements were made in this investigation. Further studies have examined the effect of dietary cholesterol within diets enriched with different in types of lipid or protein. For example, cholesterol feeding has a large depressive effect on Δ6-desaturase activity in diets rich in beef tallow but little effect in diets enriched with fish oil (Garg *et al.*, 1988a). Further work has concentrated on the viscotropic effects of cholesterol and in particular its role *in vivo* and *in vitro* on membrane bound enzymes (Brenner, 1984; Castuma and Brenner, 1986). Cholesterol enrichment of microsomal membranes *in vitro* increases Δ6-desaturase activity (Garda and Brenner, 1985) while the opposite occurs *in vivo* (Garg *et al.*, 1988a).

1.5.2 Dietary proteins and Δ6-desaturation

Diets high in protein, in the form of casein, increase Δ6-desaturase activity (Peluffo and Brenner, 1974). No additional stimulation, however, is shown above 35 % (w/w) protein. In contrast, diets low in protein (<5 % w/w) evoke low activities of Δ6-desaturase (De Tomás *et al.*, 1980; Narce *et al.*, 1988b). Further studies have examined the effects of different types of protein on Δ6-desaturative capacity. Rats fed casein supplemented diets possess a higher Δ6-desaturase activity than those fed a

soybean based diet (Choi *et al.*, 1989; Lindholm and Eklund, 1991). In addition, 20:3 n-9 is raised in liver microsomal phospholipids of rats fed casein with an adequate amount of dietary 18:2 n-6. The mechanism for these phenomena are not entirely understood. However, it may relate to the arginine content of the dietary protein (Koba *et al.*, 1993) or possibly the effect different dietary proteins have on microsomal cholesterol levels (Koba *et al.*, 1993). Interestingly, the effects of casein are also demonstrable in diets supplemented with cholesterol (Lindholm and Eklund, 1991).

1.5.3 Dietary metal ions and Δ6-desaturation

A small number of divalent cations have been studied in the context of modified tissue fatty acid compositions and altered desaturase activities. Zinc has been implicated in the regulation of EFA metabolism and interestingly humans with diseases associated with zinc deficiency also display altered tissue fatty acid profiles. Experimental studies by Cunnane (1982) have shown that tissue 18:2 n-6 content is increased in EFA supplemented, zinc deficient rats which has led to the suggestion that Δ6-desaturation capacity may be impaired. Results on tissue Δ6-desaturase activities, however, show disparities. Wahle (1983) has demonstrated that zinc deficiency does not alter hepatic microsomal Δ6-desaturase activity. Yet, Tsai *et al.* (1983) have shown that Δ6-desaturase activity is depressed in zinc deficient rats fed *ad libitum*. Other studies have shown that dietary zinc deficiency reduces testicular microsomal Δ 6-desaturase activity which is consistent with some of the fatty acid compositional changes (Clejan *et al.*, 1982). In agreement with these latter findings are the palliative effects of 18:3 n-6 in zinc deficiency (Cunnane and Horrobin, 1980).

Magnesium deficiency produces hypercholesterolaemia and other lipid metabolism defects in both humans and rats. Patients with latent tetany present increased levels of 18:2 n-6 and depressed levels of 20:3 n-6 and 20:4 n-6 in plasma phospholipids. These observations are in accord with decreased Δ6-desaturase activities and altered tissue fatty acid patterns in rats pair-fed magnesium deficient diets (Mahfouz and

Kummerow, 1989). Low Δ6-desaturase activities are also a feature of porcine kidney cells cultured in low magnesium concentrations (Mahfouz *et al.*, 1989).

1.6 Hormonal modification and Δ6-desaturase activity

A wealth of information has been assembled over the years on hormonal action and Δ6-desaturation. Insulin is the main anabolic hormone in this context with many other hormones acting antagonistically.

1.6.1 Insulin

Rat liver microsomes from alloxan-induced diabetic rats are deficient in the capacity to desaturate 18:2 n-6 (Mercuri *et al.*, 1966; Brenner *et al.*, 1968; Castuma *et al.*, 1972). Similar observations have been documented for Δ9-desaturase (Gellhorn and Benjamin, 1964). Insulin therapy repairs the defects in both Δ9- and Δ6-desaturase activities (Mercuri *et al.*, 1966; Brenner *et al.*, 1968) but this correction is partially blocked by prior *in vivo* treatment with actinomycin D (Brenner *et al.*, 1968). The inhibitory effect of actinomycin D on RNA synthesis has led to the conclusion that insulin restores desaturase activities through enzyme induction (Brenner *et al.*, 1968). Similar findings have been documented in fasted rats treated with actinomycin D prior to refeeding (Inkpen *et al.*, 1969).

Defective Δ9-, Δ6- and Δ5-desaturase activities have equally been demonstrated in rats made diabetic using streptozotocin (Eck *et al.*, 1979; Faas and Carter, 1980). Insulin treatment (8 U/day for 2 days) corrects these desaturase activities (Faas and Carter, 1980). In agreement with this is the normalisation of microsomal fatty acid composition with the exception of 20:4 n-6 which remains reduced (Faas and Carter, 1980). Furthermore, the defect in Δ6-desaturation in streptozotocin-induced diabetes has been identified not in the associated electron transport chain but in the terminal desaturase (Eck *et al.*, 1979).

Diabetic studies using chemical agents, however, can be generally criticised. The animals are hyperphagic, polydipsic, dehydrated and very ill. Such studies also do not represent the situation in human diabetics (i.e. daily insulin injections with fluctuating glycaemia and associated long term complications). The genetically prone diabetic rat offers a better alternative for the study of diabetes on EFA metabolism. These animals suffer from retinal capillary basement membrane thickening (Chakrabarti *et al.*, 1993) and polyneuropathy (Sima *et al.*, 1992) despite regular insulin treatment. EFA metabolism in the spontaneously diabetic rat has been explored but results, all from one research group, have been inconsistent. No difference in Δ6-desaturase activity between control and BB diabetic rats was demonstrated in one study (Chanussot *et al.*, 1989) despite some changes in n-6 fatty acid composition. Other studies have shown the opposite (Mimouni and Poisson, 1990; Mimouni and Poisson, 1992).

1.6.2 Glucagon, adrenaline and associated hormones

Fasting decreases Δ6-desaturase activity and refeeding reactivates the system (Inkpen *et al.*, 1969; De Gómez Dumm *et al.*, 1970). Administration of glucagon to rats after fasting abolishes the increase elicited by refeeding (De Gómez Dumm *et al.*, 1975). Dibutyryl cAMP has the same effect (De Gómez Dumm *et al.*, 1975). Furthermore, co-administration of theophylline enhances the action of dibutyryl cAMP. Conclusions from these studies hypothesised that glucagon produces its effect either directly or indirectly by influencing cAMP.

Hormones such as catecholamines also operate through the activation of adenylate cyclase. Subcutaneous administration of large amounts of adrenaline to rats (1 mg/kg body weight) increase cAMP levels (maximal at 0.5 h) and decrease the microsomal Δ 6-desaturation of 18:2 n-6 and 18:3 n-3 (De Gómez Dumm *et al.*, 1976). Δ6-desaturation is maximally reduced 12 h after adrenaline injection ($p<0.01$) but recovers to control values between 12 and 36 h later. Lower doses of adrenaline induce a small but non-significant depressive effect (De Gómez Dumm *et al.*, 1976). The action of

adrenaline is proposed to be mediated through an increase in cAMP levels that enhance the concentration of a glucose metabolite but little more is known. Unfortunately, tissue fatty acid compositional data was not reported in either the fasting-refeeding studies or the glucagon and adrenaline work.

1.6.3 Other factors affecting Δ6-desaturation

The activity of Δ6-desaturase is modulated by other components of the endocrine system. Intraperitoneal hydrocortisone injection into rats (~50 mg/kg body weight), reduces Δ6-desaturase activity in liver microsomes with a maximal response observed 24 h after administration (De Gómez Dumm *et al.*, 1979). Synthetic corticosteroids, triamcinolone (~12 mg/kg) and dexamethasone (~5 mg/kg) reduce Δ6-desaturase activity in a similar manner but are more potent compared to hydrocortisone (De Gómez Dumm *et al.*, 1979). Dexamethasone is believed to exert its inhibitory effect on Δ6-desaturation through the synthesis of a regulatory cytosolic factor (Marra *et al.*, 1986). However, washed microsomes prepared from dexamethasone-induced rats also have reduced Δ6-desaturase activity indicating that desaturase protein synthesis is also impaired (Marra *et al.*, 1986).

The affects of thyroid hormones have also been examined in the context of Δ6-desaturase activity (Faas and Carter, 1981; Ves-Losada and Peluffo, 1993). Rats given daily intraperitoneal injections of L-triiodothyronine (10 mg/kg) for 5 consecutive days reduced microsomal Δ6-desaturase activity (Ves-Losada and Peluffo, 1993). Yet, changes in microsomal fatty acid composition did not parallel this impaired Δ6-desaturase activity.

The effects of more physiological stimuli have also been examined. A high Δ6-desaturase activity is present in the neonatal rat which generally declines with age (Bourre *et al.*, 1990; Bordoni *et al.*, 1988; Biagi *et al.*, 1991). However, Maniongui *et al.* (1993), do not support this ageing effect and demonstrated no difference in Δ6-desaturase activity between rats aged 1.5 and 24 months. In contrast with this are the

results of Ullmann *et al.* (1991a) who reported an age-related increase in Δ6-desaturase activity. It is possible that some diets prevent an age associated decline in Δ 6-desaturase activity (Biagi *et al.*, 1991).

Diurnal and seasonal variations have also been documented. Diurnal changes are believed to reflect feeding behaviour (De Gómez Dumm *et al.*, 1984) and are clearly important in the planning of experiments. The documented seasonal effects on Δ6-desaturase activity may be dubious, however, as temperatures within the animal laboratory were not constant and sometimes reached over 32°C (Peluffo and Brenner, 1974).

1.7 Human studies

1.7.1 Δ6-desaturase in humans

Obviously studies on Δ6-desaturase activity in human liver are scarce due to the lack of readily available material. Activities for adults range between 7 and 125 pmol/min/mg microsomal protein (De Gómez Dumm and Brenner, 1975; Biagi *et al.*, 1990) and that of neonates is even lower (Poisson *et al.*, 1993). The necessary use of anaesthetics (De Gómez Dumm and Brenner, 1975) and the selection of patients requiring liver biopsy, implies that results from these studies may not adequately represent true activities of Δ6-desaturase in man in general. Indeed, Δ6-desaturase activities measured in a neonate study (Poisson *et al.*, 1993) used liver samples obtained 1-3 h after death, which may have also affected enzyme activities.

As a result, studies on humans often rely on tissue fatty acid compositions. These will be discussed in the context of coronary heart disease below.

Table 1.6 *Liver microsomal Δ6-desaturase activities in humans*

Study	Δ6-desaturase activity #
Human (neonate) [a]	5-8
Human (cholecistectomy patients) [b]	125
Human [c]	7-60

pmol/min/mg microsomal protein. a Poisson *et al.* (1993), b Biagi *et al.* (1990), c De Gómez Dumm and Brenner (1975).

1.7.2 Past and present trends of CHD

Coronary heart disease (CHD), ignored for much of the 19th century and before, is now recognised as one of the main causes of death in men and women within industrialised countries since the 1920's. In the UK, a peak in the mortality rate was reached in the late 1960's but mortality rates have since declined in England and Wales across all age ranges in recent years (Table 1.7). Nevertheless, the epidemic remains to be one of the leading causes of death afflicting men in their peak years of economic

activity. On an international scale, WHO figures demonstrate the condition's severity with approximately 1 in 3 (or 2.4 million) people dying from the disease in the developed world in 1987 (Lopez, 1990).

Table 1.7 *CHD mortality rates for men aged 35-74 over the last decade in England and Wales*

	Age band			
Census year	35-44	45-54	55-64	65-74
1983	443	2330	6824	15851
1984	402	2176	6777	15557
1985	414	2150	6700	15611
1986	406	2093	6397	14893
1987	398	1938	6192	14501
1988	352	1816	5899	14011
1989	355	1658	5460	13307
1990	356	1537	5206	13180
1991	335	1468	4967	12806
1992	314	1369	4723	12417
Reduction 1983-92 (%)	29	41	31	22

Values expressed per million population. Source: Office of Population, Censuses and Surveys 1983-1992

1.7.3 Risk markers and CHD

More than 240 risk markers have been associated with CHD (Hopkins and Williams, 1981). Even though the disease is believed to be multifactorial in origin, epidemiological studies cannot prove causal relations and some of these markers may be spurious and/or trivial (McCormick and Skrabanek, 1988). Established risk markers include elevated serum cholesterol (Kannel *et al.*, 1971, Kannel *et al.*, 1986), increased blood pressure (Kannel *et al.*, 1981), smoking (Doll and Peto, 1976; Townsend and Meade, 1979), age (Kannel, 1976), male gender (McGill and Stern, 1979) and diabetes (Brownlee and Cahill, 1979, Stamler *et al.*, 1993). More recently, low levels of dietary EFA have been associated with the disease (Logan *et al.*, 1978; Riemersma *et al.*, 1986).

1.7.4 Dietary EFA and CHD

Hugh Sinclair stated in his *Lancet* article in 1956 that diets high in saturated or unnatural fats were in fact low in EFA (Sinclair, 1956). Interest at that time was generally focused upon SFA and their involvement in the pathogenesis of CHD through raised serum cholesterol levels (Keys *et al.*, 1957). Sinclair's postulate was that diseases prevalent in Western civilised countries such as atherosclerosis, coronary thrombosis and cancer were due to a moderate EFA deficiency (Sinclair, 1956). Since this time many studies have been undertaken to assess whether such a dietary deficiency exists.

Dietary fatty acid intakes are difficult to assess and as a consequence alternative methods are often used. Adipose tissue triacylglycerols are a reliable indicator of long term EFA intake (Dayton *et al.*, 1966; Wood *et al.*, 1984; Katan *et al.*, 1991). Plasma cholesterol ester and triacylglycerol fatty acid composition are also used as indicators of EFA intake (De Backer *et al.*, 1989).

Pioneering studies by Kingsbury *et al.* (1962) demonstrated that adipose tissue diene content (mainly 18:2 n-6) was lower in patients with atherosclerotic disease compared with healthy controls. Furthermore, serum cholesterol esterified trienes were raised in these patients. The analytical technique used in this era, prior to gas liquid chromatography, did not permit a more in-depth assessment of triene composition but it is apparent from further studies by this group that 20:3 n-9 was the main component (Kingsbury *et al.*, 1974). This was therefore indicative of a moderate EFA deficiency and was possibly the first identification of raised 20:3 n-9 in patients with a low 18:2 n-6 content. The study, however, can be criticised as patients were of mixed aetiology (angina, aortic aneurysm, ischaemic pain and coronary thrombosis) and no adjustments were made for confounding factors such as smoking and other risk factors.

1.7.5 Population studies

The Edinburgh-Stockholm study was the first formal investigation establishing a significant association between low adipose tissue 18:2 n-6 concentration and CHD mortality rates (Logan *et al.*, 1978). It was devised to identify factors that might explain the high CHD mortality and morbidity in Edinburgh which is three times greater than in Stockholm. The study, carried out in 1976, was conducted on randomly selected healthy men aged 40. Edinburgh men had lower levels of cholesterol esterified 18:2 n-6 ($p<0.001$) although total cholesterol levels were not different. Adipose tissue triacylglycerol 18:2 n-6, a biomarker for 18:2 n-6 intake, was also significantly lower in Edinburgh men (7.3±1.5 *vs.* 11.8±2.1 %, $p<0.001$). Known classical risk factors such as LDL and total cholesterol, smoking and obesity could not explain the difference in CHD mortality between the two populations.

The above findings were confirmed by a cross-cultural study in apparently healthy men (aged 40-49) from four European regions (Riemersma *et al.*, 1986). Adipose tissue 18:2 n-6 contents were as follows: North Karelia (Finland) 7.4 %, South West Finland 8.1 %, Edinburgh (Scotland) 8.8 % and Sapri (Italy) 13.5 %. These results correlated inversely with CHD mortality rates (Fig. 1.5). In addition, the desaturation and elongation product 20:3 n-6 was also inversely associated with CHD mortality rates (Fig. 1.5) suggesting that the metabolism of 18:2 n-6 through Δ6-desaturase may be impaired in subjects with CHD. Furthermore, the difference in the proportion of 18:2 n-6 in adipose tissue between the four regions remained significant after adjustment for classical CHD risk factors. It is important to note that similar differences in CHD mortality rates and serum 18:2 n-6 content have also been demonstrated in randomly selected men in Finnish regional studies (Nikkari *et al.*, 1983).

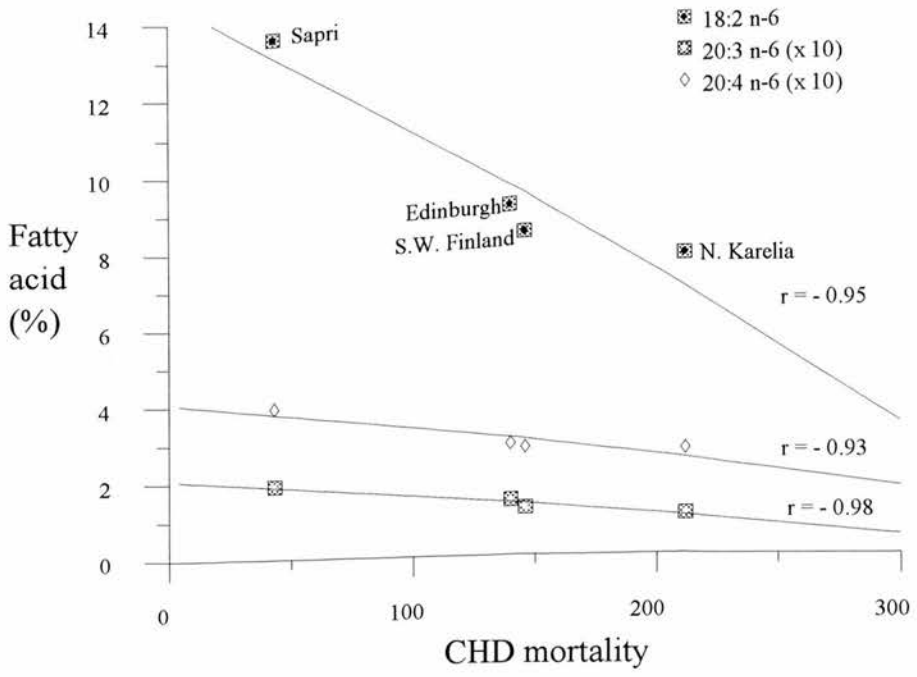


Figure 1.5 *Correlations of adipose tissue n-6 EFA content and CHD mortality from four European regions (Data from Riemersma et al., 1986)*

Other epidemiological studies support some of these findings. The Scottish Heart Health Study, a cross-sectional survey of coronary risk factors in 4144 Scottish men and women, evaluated associations between adipose tissue EFA and CHD mortality (Tavendale *et al.*, 1992). The mean adipose tissue 18:2 n-6 content, from each of the twenty-two regions studied, was inversely correlated with the standard mortality ratio (SMR) for CHD in both men and women (r=-0.60 and -0.62, respectively). In contrast, levels of 20:3 n-6 showed no association with the SMR. Wahle *et al.* (1991) also document comparably low 18:2 n-6 concentrations in another coronary prone population (Aberdeen) but values for 20:3 n-6 were not reported.

1.7.6 Case-control studies

Early case-control studies on atherosclerotic and normal individuals measured relative amounts of EFA esterified with plasma cholesterol (Lewis, 1958; Björntorp, 1960; Schrade *et al.*, 1961; Böttcher and Woodford, 1961). In general these studies

documented lower levels of 18:2 n-6 and 20:4 n-6 in the atherosclerotic individuals compared with controls. However, criticism can be levelled at these studies. For example, the controls used by Böttcher and Woodford (1961) were 30 years younger than the cases, who were of mixed aetiology. Finally, the possibility that these subjects might have changed their diets after the diagnosis was not always examined.

A cross-sectional study in the East of Scotland avoided this problem by detecting men with previously unidentified or occult CHD (Wood *et al.*, 1984). The study entailed an assessment of adipose tissue and platelet fatty acid composition in a total random sample of 448 men. Retrospective analysis showed that 28 had occult CHD and therefore had not contacted their doctors regarding lifestyle and dietary changes. Lower concentrations of adipose tissue 18:2 n-6 content were demonstrated in these new cases compared with men with no CHD (n=343). In addition, low levels of adipose tissue 20:3 n-6 were more significantly related with new CHD than 18:2 n-6 suggesting a possible impairment of EFA metabolism as the most important factor (Oliver *et al.*, 1989).

This study was followed by a prospective case-control investigation of angina and acute myocardial infarction. There was an inverse relative risk of new angina pectoris and acute myocardial infarction (AMI) according to the concentration of 18:2 n-6 in adipose tissue (Wood *et al.*, 1987). However, in contrast to the above retrospective study no differences were observed for the concentration of either 20:3 n-6 or 20:4 n-6 in adipose tissue. There is no satisfactory explanation for this discrepancy between the two studies of Wood *et al.*

1.7.7 Prospective studies

One of the main problems with case-control studies is that measurements are made after the onset of the disease and it cannot be ascertained therefore whether the differences are a cause leading to or promoting disease development or an effect of the

disease itself. Prospective studies have the advantage over typical case control studies because dietary analyses are made long before the disease is manifest.

An assessment of the predictive power of serum fatty acid composition in the development of CHD formed the basis of a prospective study by Miettinen *et al.* (1982). Briefly, 1222 men, initially free from CHD, were followed-up for 5 to 7 years. During this time 33 sustained fatal AMI, non-fatal AMI or sudden death. The relative amount of phospholipid esterified 18:2 n-6, a poor marker of dietary 18:2 n-6 intake, was discovered to be an independent risk factor for CHD. The absence of an inverse association between the percentage of cholesterol esterified 18:2 n-6 (which does reflect dietary 18:2 n-6) and subsequent AMI, was not different between patients with infarcts and the retrospectively selected controls.

A number of other prospective studies have been undertaken assessing the dietary intakes of PUFA in association with CHD mortality (Gordon *et al.*, 1981; McGee *et al.*, 1984; Kushi *et al.*, 1985; Kromhout and Coulander, 1984). The findings from these studies in different populations have also been inconsistent.

1.7.8 Intervention studies

CHD intervention studies can be classified as either primary or secondary trials. Primary trials are established to prevent end points of CHD (such as angina, MI or death) in people who are at risk but are nevertheless still free from discernible disease. In contrast, secondary intervention trials are aimed at the prevention of the occurrence of a second MI or similar cardiac related event.

Only a small number of primary intervention studies have solely examined the effect of dietary change in connection with CHD. The randomised and double-blinded, Los Angeles Veterans study (Dayton *et al.*, 1969), demonstrated a decrease in coronary events in men fed diets with a high P/S ratio. Yet, the effect was only significant in those who had an initially high serum cholesterol. The study, however, is associated with a number of uncertainties. For example, a high non-CHD mortality rate

was recorded in those who received the experimental diet. In addition, adherence to either the control or experimental diet was poor (Gurr, 1992).

The Finnish mental hospitals study also examined the effect of dietary change on CHD mortality using a dietary protocol similar to the Veterans study (Turpeinen *et al.*, 1979). Patients were recruited from two mental hospitals and received either their usual diet or a modified diet for a period of six years. Despite a large reduction in serum cholesterol and a generally favourable risk profile, deaths from CHD remained unchanged. Unfortunately, this may have been due to the changeable study population as a result of new admissions and discharges.

Other CHD intervention trials have concentrated upon changing a broad range of risk factors (Rose *et al.*, 1980; Hjermann *et al.*, 1981; Neaton and Wentworth, 1992). Indeed, the study of Neaton and Wentworth (1992) is more commonly known as the Multiple Risk Factor Intervention Trial. As their name suggests, however, they examine a whole variety of risk factors and as such the contribution of one element, namely dietary 18:2 n-6, is uncertain.

1.7.9 Other EFA studies in humans

The finding that low levels of adipose tissue 20:3 n-6 reflected regional gradients of CHD mortality (Riemersma *et al.*, 1986) prompted Abraham *et al.* (1990) to examine whether an impaired Δ6-desaturase was important in an Edinburgh based CHD prone population. Apparently healthy men with a low adipose tissue 20:3 n-6 content, that had previously taken part in an earlier study (Wood *et al.*, 1987), were selected. Adipose tissue and serum lipid fatty acid compositions were analysed before and after a four month supplementation with evening primrose oil (EPO, containing 9 % 18:3 n-6) or saffower oil (containing no 18:3 n-6) as control. EPO but not safflower increased adipose tissue 20:3 n-6 content. Similar qualitative changes were observed in both serum triaclyglycerols and cholesterol esters. The results suggested that 18:2 n-6 is not readily converted to 20:3 n-6 and this may be due to a low activity

of Δ6-desaturase in these highly selected men. A similar type of study is required in a population with a low rate of CHD mortality, such as that of Southern Italy.

In a small study of two patients with hyperlipidaemia and two controls, the conversion of an orally administered dose of [1-^{14}C]18:2 n-6 was investigated by Nichaman *et al.* (1967). Blood samples were taken at two hour intervals after the dose and plasma radioactivity determined. The conversion [1-^{14}C]18:2 n-6 to 20:3 n-6 and 20:4 n-6 in plasma phospholipids was limited and shown to be lower in hyperlipidaemics than controls. Other studies investigating the effects of risk factors on 18:2 n-6 metabolism in humans are scarce.

1.8 Summary

Low levels of 18:2 n-6 in adipose tissue are associated with increased risk of CHD mortality. Decreased quantities of the desaturation and elongation product 20:3 n-6 also reflect CHD mortality gradients and suggest a role for an impared 18:2 n-6 metabolism at the level of Δ6-desaturase. Evidence from animal studies have shown that certain factors change the activity of Δ6-desaturase and some of these modulators are also risk markers for CHD. What is unkown is how these factors alter the fatty acid composition of tissues that are used to determine risk of CHD in individuals.

Hypothesis

This thesis evaluates the effects of different modulators of Δ6-desaturase activity and assesses how fatty acid composition is altered by these modulators in liver, plasma and adipose tissue. It postulates that the activity of the Δ6-desaturase is reflected in the fatty acid composition of the tissues measured.

Chapter 2
Materials and Methodology

2.1 Animal experiments

Male, Sprague Dawley rats (~100 g, 4-5 weeks old), purchased from Bantin and Kingman Company (Kingston-upon-Hull, Humberside, U.K.), were employed in the majority of the dietary experiments (Chapters 4, 5 and 6). They were housed in groups of four (unless otherwise stated) at a constant temperature (21±1°C), with water available *ad libitum* and a cycle of 12 h dark, 12 h light (0800-2000 hours). Free access to food (dependent on experiment type) was given unless stated to the contrary.

Diabetic studies (Chapter 7) were performed on male, Wistar Bio Breeding rats (300-400 g, 20-23 weeks old) resident in the Biomedical Research Facility of the Western General Hospital, Edinburgh. Rats were housed in groups of four at ambient temperature (20±1°C) with a 12 h dark/light cycle (0800-2000 hours). They were provided food (Rat & Mouse No.1 (Modified), S.D.S., Witham, Essex, U.K.) and water *ad libitum*. Daily subcutaneous injections of bovine insulin (Ultratard, Novo Nordisk, Copenhagen, Denmark) were given to diabetic rats at 1000 hours. The dose was adjusted for each animal individually according to daily measurements of body weight and the degree of glycosuria (Chapter 7).

2.2 Experimental diet formulation

The diets for the cholesterol feeding studies (Chapters 4, 5 and 6) were semi-synthetic. The control diet was prepared from the basic dietary components of casein, cornflour, glucose, beef tallow, safflower oil, non-nutritive cellulose, L-methionine, salt mix (Appendix A) and vitamin mix (Appendix B). The constituents were combined to give 38.4 % of total energy from fat, 38.0 % from carbohydrate and 23.6 % from protein (Appendix C). Diets were prepared by combining the dry ingredients (including salt and vitamin mixes) in a commercial food mixer (Robot Coupe) and blending

thoroughly using the low speed setting for two minutes. The fat mix (comprising molten beef tallow (liquefied in a Microwave oven (Toshiba, model ER-7820EW, Tokyo, Japan)) and safflower oil with 106 mg supplemental (+)-α-tocopherol acetate; Appendix C) was added slowly to the dry mix preparation at low mixing speed and then blended at high speed for two minutes. The mineral and vitamin quantities given in the diets were derived from Unilever Research, Vlaardingen, Holland.

To minimise potential peroxidative effects on dietary PUFA, diets were not hydrated or pelleted, prepared weekly and stored at <4°C under argon in sealed polythene containers. Fresh diets were supplied on the same days that animal cages were changed (i.e. Monday, Wednesday and Friday of each week) with used diet being discarded.

2.3 Tissue collection and preparation

2.3.1 Liver

Rats were sacrificed (normally between 0900 and 1000 hours) by stunning followed by cervical dislocation (unless otherwise stated). Livers were quickly excised and rinsed in ice cold homogenisation buffer (0.25 M sucrose, 1 mM dithiothreitol, 1 mM disodium EDTA, 0.1 M potassium dihydrogen orthophosphate, pH 7.2). The liver from each rat was weighed individually and minced finely with scissors on an ice-cold petri-dish. The tissue was then homogenised with five volumes of the above buffer in a Dounce tissue grinder (Wheaton, Millville, New Jersey, U.S.A.) with ten up and down strokes of loose and tight plungers respectively. The homogenate was centrifuged for 10 min at 800 × g in a Beckman rotor (JA 14). The resultant pellet was discarded and the supernatant was centrifuged for 20 min at 15,000 × g (Beckman rotor Ti 45). The microsomal pellet was obtained by centrifugation of the 15,000 × g supernatant at 105,000 × g for 60 min (Ti 45). The pinkish pellet that resulted was gently reconstituted in a volume of homogenisation buffer in proportion to the starting weight of the tissue (0.1 ml/g liver). The samples were stored at -20°C until analysis

(within four weeks). All centrifugation steps were carried out using the Beckman J2.21 centrifuge and the Beckman L8-55 ultracentrifuge at 4°C (Beckman Instruments Inc., Palo Alto, U.S.A.) according to Garg *et al.* (1988a).

2.3.2 Blood, heart and adipose tissue

Just prior to liver excision, blood was taken by cardiac puncture, collected in lithium heparin tubes (Sterilin Ltd., Teddington, Middlesex, U.K.), centrifuged (800 × g) and plasma stored (-20°C) for biochemical analyses. The heart was then excised, rinsed in ice-cold saline, weighed and frozen in liquid nitrogen. Adipose tissue from the inguinal region of each rat was also removed, rinsed in ice cold 0.9 % (w/v) saline and stored (-40°C).

2.4 Biochemical analyses

2.4.1 Plasma cholesterol and triacylglycerol

Plasma cholesterol and triacylglycerol were determined enzymatically (CHOD-PAP, Cholesterol C system, Boehringer Mannheim, Germany and Merkotest 14360, Merck, Germany). Both assessments were carried out using a Cobas Bio centrifugal analyser (Roche Diagnostics, Welwyn Garden City, Hertfordshire, U.K.). Details are presented in Appendix D. The intra- and inter- assay coefficients of variation for the cholesterol analyses were 0.8 and 4.9 %, respectively. The corresponding coefficients for the triacylglycerol analyses were 1.1 and 1.5 %, respectively.

2.4.2 Plasma insulin

Plasma insulin was determined using radioimmunoassay with rat insulin standards (Novo Research Institute, Bagsvaerd, Denmark) and charcoal separation (Ashby and Speake, 1975). This assay was performed courtesy of Dr R.M. Lindsay, Principal Biochemist, Metabolic Unit, Western General Hospital, Edinburgh. The intra- and inter- coefficients of variation for insulin analyses were 4.2 and 7.3 %, respectively.

2.4.3 Plasma glucose

Plasma glucose analyses were performed at the Western General Hospital using a Beckman Synchron AS4 automated analyser system (Beckman-RIIC, High Wycombe, Buckinghamshire, U.K.) employing the glucose oxidase method (Swoboda and Massey, 1965). The intra- and inter- coefficients of variation for this assay were 0.8 and 2.2 %, respectively.

2.4.4 Glycosylated haemoglobin

Red blood cells were washed three times in isotonic saline on the day of sample collection and then frozen (-20°C). Glycosylated haemoglobin was analysed at the Western General Hospital using boronic acid affinity chromatography (Pierce and Warriner, Chester, Cheshire, U.K.). The intra- and inter- coefficients of variation were 1.9 and 3.4 %, respectively.

2.4.5 Microsomal protein

Analysis of microsomal protein concentration was carried out by the method of Lowry *et al.* (1951) modified by Clifton *et al.* (1988) for estimation on the Cobas Bio centrifugal analyser fitted with the DENS calculation programme (Roche Diagnostics, Welwyn Garden City, Hertfordshire, U.K.). Bovine serum albumin (Fraction V) dissolved in homogenisation buffer was the protein standard solution. The start reagent was Folin and Ciocalteu's phenol reagent (diluted 1:3 (v/v) with double distilled water) and the absorbance measured at 750 nm. The standards 0, 100, 200, 300, 400 and 500 µg/ml were run within each estimation and the sample protein concentration calculated through the DENS plot mode. The inter- and intra- coefficients of variation were 2.9 and 5.7 %, respectively.

2.4.6 Microsomal succinate dehydrogenase

Succinate dehydrogenase (SDH) activity was assessed by the ability of microsomes and total homogenate to reduce 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-tetrazolium chloride (INT) in the presence of phenazine methosulphate (PMS) (Porteous and Clark, 1965). The incubations (1.0 ml) contained (in μmoles) sodium phosphate, 50.0; sodium succinate, 10.0; INT, 0.40; PMS, 0.33 and Triton X-100, 0.0001 %. Microsomes and total homogenate (2 mg protein) were assayed. Electron acceptors were added after a 6 min pre-incubation (30°C). Absorbance increases were followed at 540 nm on a SP 8-100 ultraviolet spectrophotometer (Pye Unicam). The reference incubation contained 10 μmoles sodium malonate instead of sodium succinate. An absorbance increase of 0.1 represented a reduction of 1.77×10^{-8} moles INT (assuming a transfer of 2 electrons per INT molecule). SDH activity was expressed as nmol/min/mg protein. Mitochondrial contamination of microsomes was expressed as a percentage of the activity of SDH in microsomes of SDH in the total homogenate.

2.4.7 Adenosine triphosphate and creatine phosphate

ATP and CP concentrations were determined using the enzymatic ultraviolet method of Trautschold *et al.* (1985) adapted for use on a Cobas Bio centrifugal analyser (Wardle, 1990). Details are provided in Appendix D.

2.4.8 Δ6-desaturase

Liver microsomal Δ6-desaturase activity was analysed in groups according to the design of the individual experiments (n=16-24) with a blank measured alongside. For larger experiments (Chapter 6), the analyses were paired (i.e. control and experimental microsomes from the same study were measured in the same analytical run) with a quality control and blank assessed alongside.

Oxidative desaturation of 18:2 n-6 by liver microsomes was estimated by measuring the amount of [1-^{14}C]18:3 n-6, [1-^{14}C]20:3 n-6 and [1-^{14}C]20:4 n-6 produced from [1-^{14}C]18:2 n-6 (Brown and Riemersma, 1995, submitted for publication). The freshly prepared incubation medium contained in a total volume of 1.2 ml the following compounds (in μmoles): ATP, 4.0; Coenzyme A, 0.25; NADH, 1.25; nicotinamide 0.5; magnesium chloride 5.0; bovine serum albumin (essentially fatty acid free) 0.01; glutathione 1.5; potassium phosphate 45.0 (pH 7.2) and 200 nmoles of [1-^{14}C]18:2 n-6 (CFA 104, Amersham International, Amersham, Buckinghamshire, U.K.) dissolved in 4.2 μl ethanol. The substrate was diluted with unlabelled 18:2 n-6 to produce a specific activity of 700 dpm/nmol.

Incubations were commenced by the addition of 500 μg of microsomal protein (in 200 μl homogenisation buffer) to 1.0 ml of pre-warmed (37°C) incubation medium in a Quick-fit tube (18 mm × 125 mm). The desaturation preparation was quickly vortexed (1-2 s) and placed back into the reciprocal shaking (120 cycles/min) water bath (37°C) for 20 min. The reaction was terminated by the addition of 2.0 ml of 10 % (w/v) KOH in 80 % (v/v) aqueous methanol containing 0.005 % (w/v) butylated hydroxytoluene (BHT). Lipids were saponified (45 min, 82°C, under argon), cooled to room temperature and acidified with 1.0 ml 7 M HCl, the non-esterified fatty acids were then extracted into hexane (4.0 ml, 0.005 % (w/v) BHT). The hexane extract was evaporated under argon (British Oxygen Company (B.O.C.) Ltd, Guildford, Surrey, U.K.) in an aluminium block at 30-40°C (Wess, Technischer Werkstätten, Germany) and methylated by heating in an oven (100°C, 60 min) with 1.0 ml 14 % (w/v) BF_3-methanol reagent under argon. The methylation mixture was then cooled to room temperature, 4.0 ml distilled water was added and the fatty acid methyl esters extracted with 2.0 ml hexane (0.005 % (w/v) BHT). Carrier methyl esters of 18:2 n-6, 18:3 n-6 and 20:4 n-6 were added to the hexane extract and evaporated to dryness using a Büchi R rotary evaporator (Orme Scientific Ltd., Manchester, U.K.) at 30°C. The extract was resuspended in chloroform (100 μl) and applied to silica gel 60 TLC

plates impregnated with 10 % (w/v) silver nitrate (Section 2.5.8). Plates were developed in chloroform-methanol-acetic acid-water (90:7.5:7.5:0.8, v/v/v/v, Gardiner and Duncan, 1991) in solvent tanks lined with filter paper (20 by 20 cm, Whatman International Ltd, Maidstone, Kent, U.K.) for separation of dienes (methyl linoleate, Rf=0.91) from trienes (methyl-γ-linolenate and methyl dihomo-γ-linolenate, Rf=0.79) and tetraenes (methyl arachidonate, Rf=0.76). No attempt was made to separate methyl-γ-linolenate from methyl dihomo-γ-linolenate. The bands were made visible under ultraviolet light (365 nm) after light spraying with 2',7'-dichlorofluorescein (0.1 % (w/v) in 50 % (v/v) aqueous methanol) and scraped directly into scintillation vials (25 mm × 58 mm, Sarstedt, Leicester, Leicestershire, U.K.). Methanol (0.5 ml) was added to elute the fatty acids from the scraped silica bands and samples were counted with 10 ml scintillation fluid (0.125 % (w/v) 2-(4-t-butyl phenyl)-5-(4-biphenylyl)-1,3,4-oxadiazole (butyl-PBD) in toluene) using a liquid scintillation counter (Model 1209 Rackbeta, Wallac Oy, Finland) for 20 min with external standardisation.

The percentage desaturation was calculated as the ratio of the counts in the desaturation products (triene plus tetraene fatty acid methyl esters) to the total counts (sum of the counts in the substrate plus products). The number of pmol of products formed were then calculated. Enzyme activity was expressed as pmol of products formed from 18:2 n-6 per min per mg of microsomal protein. Average recovery of radioactivity was 78±4 %. The intra- and inter-coefficients of variation were 3.1 and 4.8 %, respectively.

2.4.9 Δ9-desaturase

Oxidative desaturation of 18:0 was estimated by measuring the amount of [1-^{14}C]-18:1 n-9 formed during the incubation of liver microsomes with [1-^{14}C]18:0 (CFA 24, Amersham International, Amersham, Buckinghamshire, U.K.). The cofactors in the incubation were the same as those described for 18:2 n-6 desaturation except that half the amount of albumin (essentially fatty acid free) was used as the amount of substrate

utilised was 100 nmol per 1.2 ml. The same saponification, extraction and methylation procedures were used as described in Section 2.4.8. Carrier methyl esters of 18:0 and 18:1 n-9 were used to facilitate the isolation of the saturated and monounsaturated radioactive methyl esters. Plates were developed in hexane-diethyl ether (95:5, v/v; Christie, 1982) and visualised under ultraviolet light after spraying lightly with 2',7'-dichlorofluorescein. Bands were scraped from the plates into vials and counted with scintillation fluid (as described in Section 2.4.8).

The percentage desaturation of 18:0 was calculated as the ratio of counts in the monounsaturated band to the total counts (sum of counts of saturated plus monounsaturated methyl esters). The number of pmol of 18:1 n-9 formed was then calculated. The enzyme activity was expressed as pmol of 18:1 n-9 formed from 18:0 per min per mg of microsomal protein.

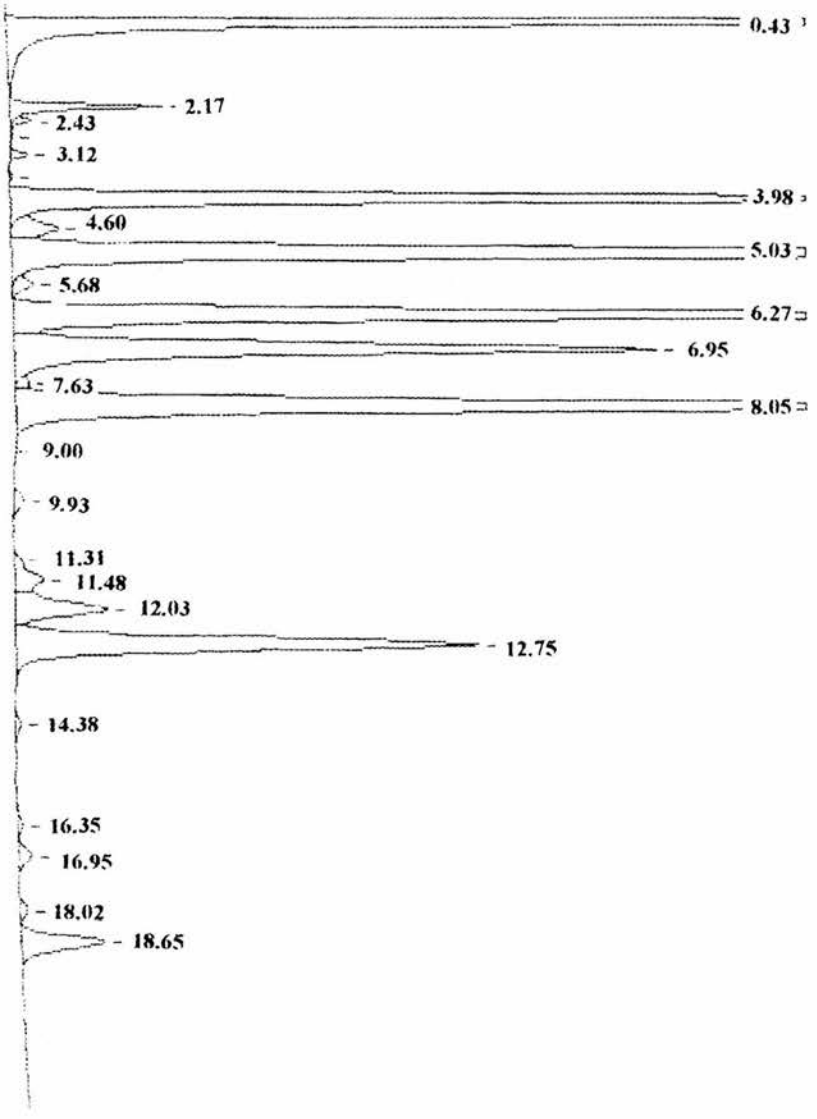
2.5 Chromatographical analyses

2.5.1 Gas liquid chromatography

All fatty acid methyl ester quantifications were carried out on a Pye Unicam series 204 chromatograph fitted with an auto-injector (Pye Unicam 4700) and linked to a Perkin Elmer Nelson 900 series interface (Perkin Elmer Nelson Systems, Inc., Cupertino, U.S.A.) and a Perkin Elmer Nelson integration system (Model 2600, 5.1) on an IBM Personal System 2 computer (Model 30, 286). A 1.5 m long glass column (i.d. 2 mm) packed under vacuum with a stationary phase of GP 10 % SP 2330 on 100/120 mesh chromosorb® WAW (Supelchem, Saffron Walden, Essex, U.K.) was used in all separations. The parameters used for the separation of one µl injected samples were as follows:- injector temperature 220°C; column temperature 180°C; detector temperature 300°C; carrier gas (oxygen free nitrogen, B.O.C. Ltd, Guildford, Surrey, U.K.) flow 50 ml/min.; hydrogen gas (B.O.C. Ltd, Guildford, Surrey, U.K.) flow 50 ml/min.; air flow 550 ml/min. A single step oven temperature programme was employed for the separation of the fatty acid methyl esters. It was composed of: 180°C

for 3 min then 3°C/min up to 250°C and 5 min at 250°C. The chromatograms were individually examined on the video screen for correct separation, retention times and base line tracking of the fatty acid peaks. Samples were reanalysed where necessary on a Perkin Elmer Nelson integration system.

A typical trace of fatty acid methyl esters derived from a rat plasma phospholipid sample is shown in Figure 2.1. The intra-coefficient of variation for individual fatty acids was dependent upon the size of the peak involved. In the analysis of adipose tissue triacylglycerols for example, the coefficient of variation for larger peaks (e.g. 18:1) was 0.6 % while for smaller peaks (e.g. 20:3 n-6) the coefficient of variation was 3.1 %.



Retention time (min) ‡	Fatty acid methyl ester	Retention time (min)	Fatty acid methyl ester	Retention time (min)	Fatty acid methyl ester
2.43	14:0	9.00	18:3 n-6 #	12.75	20:4 n-6
3.98	16:0	9.17	18:3 n-3	14.38	20:5 n-3
4.60	16:1	9.93	20:1	16.35	22:4 n-6
6.27	18:0	11.31	20:6 n-6	16.95	22:5 n-6
6.95	18:1	11.48	20:3 n-9	18.02	22:5 n-3
8.05	18:2 n-6	12.03	20:3 n-6	18.65	22:6 n-3

‡ Peak at 0.43 min is injection solvent, peak at 2.17 min is BHT, peak at 5.03 min is 17:0 (internal standard). # Unresolved peak contained unspecified amounts of 20:0.

Figure 2.1 *A typical GLC trace of plasma total phospholipid fatty acid methyl esters*

2.5.2 Fatty acid composition - liver microsomes

Liver microsomal lipids were analysed in groups according to the design of individual experiments (n=16-24) with a blank assessed alongside. However, for larger experiments (Chapter 6) paired analyses were performed so that the control microsomes were run with the experimental samples in each batch.

Liver microsomes (8-10 mg) were thawed on ice, diluted to 1.0 ml with homogenisation buffer and extracted essentially to the method of Folch *et al.* (1957). This procedure employed shaking the sample cumulatively with 3.0 ml methanol, 3.0 ml chloroform (including heptadecanoic acid, cholesteryl heptadecanoate, L-α-phosphatidylcholine diheptadecanoyl and triheptadecanoin internal standards), a further 3.0 ml chloroform and 2.5 ml 0.88 % KCl solution in a Quick-fit tube (23 mm × 150 mm). The extraction mixture was then left overnight (4°C). Following centrifugation (800 × g, 10 min), the upper aqueous phase was aspirated and discarded, the lower chloroform layer (5.0 ml) was transferred to a clean Quick-fit tube (18 mm × 125 mm) and evaporated to dryness using a Büchi R rotary evaporator at 30°C. The extract was redissolved in 200 µl chloroform and applied as a 5 cm band to silica gel 60 TLC plates (Merck No. 5721, 20 by 20 cm, 0.25 mm, pre-run in chloroform-methanol (4:1, v/v) and re-activated for 30 min at 110°C). Plates were developed in hexane-diethyl ether-formic acid (80:20:2, v/v/v) for 50 min, dried, sprayed lightly with PPO-POPOP (1,2-bis[2-(5-phenyloxazolyl)] benzene, 0.01 % (w/v) and 2,5-diphenyloxazole, 0.006 % (w/v)) in chloroform and the relevant lipids (most commonly phospholipids but also triaclyglycerols *etc.*) identified under ultraviolet light at 365 nm. The position of the bands using this separation had been identified previously using known standards. The bands of interest were scraped from the plate onto grease-proof paper and placed into separate Quick-fit tubes (18 mm × 125 mm).

Transmethylation (base catalysed) was carried out using toluene and sodium methoxide as described by Christie (1982). The methyl esters produced from this

trans-esterification were redissolved in 25 µl chloroform (containing 0.01 % (w/v) BHT) and analysed on a packed column GLC (see Section 2.5.1).

2.5.3 Fatty acid composition - liver microsomal fractionated phospholipids

For fractionated phospholipid fatty acid analysis, microsomes were extracted for total lipids as described in Section 2.5.2. The internal standards used in this analysis were L-α-phosphatidylcholine diheptadecanoyl and L-α-phosphatidylethanolamine diheptadecanoyl. After extraction, the lipid extract was evaporated to dryness (Büchi R rotary evaporator at 30°C) and redissolved in 1.0 ml chloroform-methanol (2:1, v/v). Phospholipids were separated into PC, PE and PI by TLC (Fine and Sprecher, 1982). An aliquot (500 µl) was spotted as a 10 cm streak on Whatman LK5 plates (20 by 20 cm, 0.25 mm, preadsorbant strip) which had been pre-developed in chloroform-methanol (1:1, v/v), impregnated with 1.2 % boric acid in ethanol-water (1:1, v/v) and activated (100°C, 60 min). Plates were developed in chloroform-methanol-water-ammonium hydroxide (120:75:6:2, v/v/v/v) in filter paper lined tanks for 60 min. Plates were then air dried and sprayed lightly with dichlorofluorescein (0.1 %, w/v) to allow individual bands to be identified under ultraviolet light (365 nm). The bands of interest were scraped individually from the plate and put directly into Quick-fit tubes (18 mm × 125 mm). In the case of PI (which was not available in the diheptadecanoyl form), 4.0 µg of L-α-phosphatidylcholine diheptadecanoyl was added as an internal standard to the silica scrapings. Transmethylation, extraction and analysis of methyl esters were carried out as described in Sections 2.5.2 and 2.5.1.

2.5.4 Fatty acid composition - plasma lipids

Plasma (0.50 ml) samples were thawed on ice and diluted with 0.50 ml saline. Plasma lipids (phospholipids, cholesterol esters and triacylglycerols) were extracted, separated, transmethylated and analysed as in Sections 2.5.2 and 2.5.1.

2.5.5 Fatty acid composition - dietary lipids

An accurately weighed sample of diet equivalent to ~80 mg fat was extracted with 240 ml chloroform-methanol (2:1, v/v). A 15.0 ml aliquot was withdrawn and transferred to a Quick-fit tube (23 mm × 150 mm) containing 1.0 ml triheptadecanoin as internal standard. KCl (4.0 ml, 0.88 % (w/v)) was added and the mixture shaken and centrifuged (800 × g, 10 min). A 5.0 ml aliquot was withdrawn, transferred to a clean Quick-fit tube (18 mm × 125 mm) and evaporated to dryness using a Büchi R rotary evaporator at 30°C. Transmethylation and analysis were carried out as described in Sections 2.5.2 and 2.5.1.

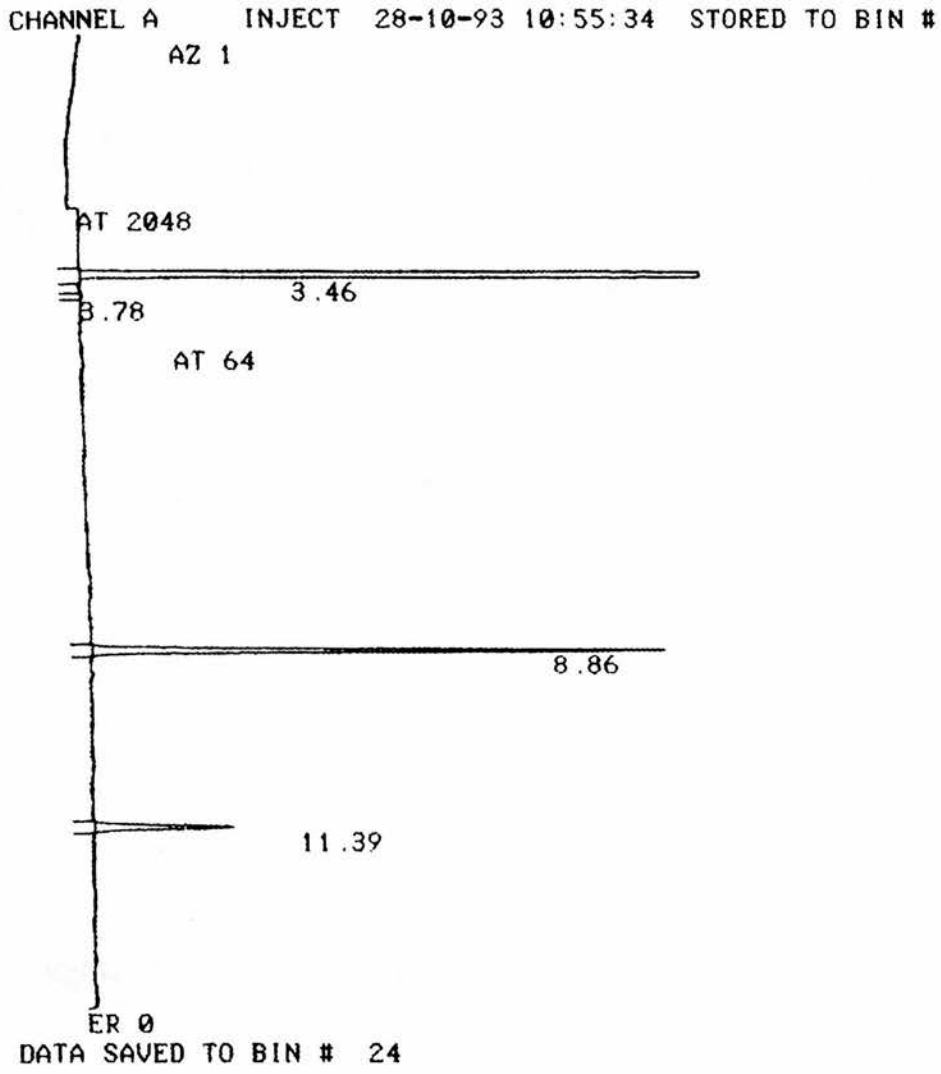
2.5.6 Fatty acid composition - adipose tissue triacylglycerols

Adipose tissue triacylglycerol was extracted essentially according to Walldius and Rubba (1976). Approximately 10 mg of adipose tissue (previously rinsed in saline solution at sample collection) was homogenised in all-glass Potter-Elvjehem homogenisers using 5.0 ml 2-propanol-heptane (4:1, v/v). Phospholipids and non-esterified fatty acids were back-extracted using 3.0 ml KOH (0.05 %, w/v) with the triacylglycerol fraction remaining in the upper organic phase. This upper phase was washed with 8.0 ml 2-propanol-KOH (0.05 % (w/v); previously shaken with heptane (4:1:3, v/v/v). The heptane layer was then transferred to a Quick-fit tube (18 mm × 125 mm) and evaporated to dryness using the Büchi R rotary evaporator. The adipose triacylglycerol fraction was transmethylated (as in Section 2.5.2) and methyl esters were extracted into hexane. The hexane was evaporated and the residue was redissolved in 40 µl chloroform for GLC analysis.

2.5.7 Cholesterol - liver microsomes

Total rat liver microsomal cholesterol content was determined according to the method of Miettenen *et al.* (1965). Microsomes (equivalent to 4-6 mg microsomal protein) were thawed and pipetted into Quick-fit tubes (18 mm × 125 mm) containing

500 μg 5-β-cholestane as internal standard (previously added in 1.0 ml 2-propanol and evaporated to dryness using a Büchi R rotary evaporator, 30°C). After alkaline hydrolysis in 10.0 ml 1 M KOH in 90 % ethanol (82°C, 60 min) the samples were cooled, filtered (Whatman No.1, Whatman International Ltd, Maidstone, Kent, U.K.) and a 5.0 ml aliquot withdrawn for extraction. Double distilled water (2.5 ml) was added and the cholesterol extracted, with shaking, into hexane (2 × 3.0 ml, then 1 × 5.0 ml hexane). The combined extract (reduced to 5.0 ml, Büchi R rotary evaporator, 30°C) was washed with double distilled water (2 × 5.0 ml) and dried with anhydrous sodium sulphate. The final hexane extract was evaporated to dryness (Büchi R rotary evaporator, 30°C). Neutral sterols were derivatised to their trimethyl silyl (TMS) ethers with 8 drops Tri-Sil® reagent (Pierce and Warriner Ltd., Chester, Cheshire, U.K.) and incubated at room temperature (30 min). The mixture was evaporated to dryness (Büchi R rotary evaporator, 30°C), resuspended in 1.0 ml n-decane, shaken for 5 min, centrifuged (800 × g, 10 min) and transferred to vials for analysis. All sterol-TMS ether analyses were carried out by GLC (Chrompack Model CP 9000 GC, Middelburg, Holland). Separations were conducted on a capillary column (25 m × 0.32 mm i.d.) with BPX as stationary phase (SIG. Pty Ltd, Milton Keynes, Buckinghamshire, U.K.) and nitrogen (B.O.C. Ltd. Guildford, U.K.) as carrier gas (split ratio 60). Initial oven temperature was 260°C increasing to 330°C, rising 5°C per min. Peak integration was performed on a Spectra-Physics SP 4600 integrator (Chrompack, Middelburg, Holland). The intra- and inter- assay coefficients of variation were 4.6 and 9.0 % respectively, when performed on liver microsomes. An example of a typical chromatogram is presented in Fig. 2.2.



Retention time (min)	Compound
8.86	5β-cholestane †
11.39	cholesterol

† Internal standard

Figure 2.2 *Typical GLC trace of rat liver microsomal cholesterol with 5β-cholestane as internal standard*

2.5.8 $AgNO_3$ thin layer chromatography plate preparation

Precoated silica gel 60 TLC plates (20 by 20 cm, 0.25 mm, No. 5721, Merck Ltd, Leicestershire, U.K.) were immersed in 10 % (w/v) silver nitrate in 80 % (v/v) aqueous ethanol for 10 s in a dipping chamber (Whatman International Ltd, Maidstone, Kent, U.K.) in low light. The plates were dried briefly in air and immediately activated (110°C, 60 min). After activation the plates were either used forthwith or stored for up to 24 h in the dark. The typical silver content of the silica gel was 7.3 % (determined by weighing plates pre and post silver nitrate impregnation).

2.6 Preparation of [11,12-^{3}H] stearic acid

2.6.1 Synthesis

The *in vivo* metabolism of [11,12-^{3}H]18:0 is dealt with in this thesis (Chapter 6). This substrate, used to assess Δ9-desaturase activity *in vivo,* is not available commercially and therefore had to be synthesised within the Unit from vaccenic acid methyl ester (11-octadecenoic acid methyl ester).

Catalytic reductions of vaccenic acid methyl ester were performed using tritiated sodium borohydride (NaB^3H_4, TRA 45, 98 % purity, 338 mCi/mmol, Amersham International, Amersham, U.K.) and a finely divided platinum catalyst on carbon, essentially according to Brown and Brown (1962) and Brown *et al.* (1963). The product fatty acid methyl esters (FAME) were hydrolysed with 30 ml 1 M KOH-ethanol (82°C, 45 min) acidified with 20 ml 6 M HCl and extracted into hexane. The extracted non-esterified fatty acids were then remethylated using BF_3-methanol (100°C, 60 min) and the resultant FAME were extracted into hexane and separated by silver ion acid-washed-Florisil™ column chromatography (Section 2.6.2). The product was analysed by GLC for purity (Section 2.5.1) then hydrolysed with KOH (1 M), acidified HCl (6 M) and extracted into hexane.

Preliminary fatty acid hydrogenations, using non-radioactive $NaBH_4$ and oleic acid methyl ester, were conducted in a similar manner as intended for the radioactive synthesis. This was due to the high cost of vaccenic acid methyl ester (£73.40/g) and to obtain relevant experience in the technique. In addition it was necessary to optimise the conditions for the reduction of the monoene.

$NaBH_4$ (3 mg) was put into the reaction vessel previously oven dried and argon flushed. Dry ethanol containing 50 mg carbon and a magnetic stirrer were added and the vessel sealed. Other reagents were added via a 100 µl syringe (Hamilton Co., Reno, Nevada, U.S.A.) through a rubber seal; platinic chloride (H_2PtCl_6, 10 mg/1500 µl (in dry ethanol), oleic acid methyl ester (880 mg), and 340 µl 6 M HCl. The reaction was allowed to proceed at room temperature for 25 min with vigorous stirring and then $NaBH_4$ (94.5 mg in 2900 µl dry ethanol) was added gradually so that the pressure within the vessel remained constant (assessed with a water filled manometer). Samples for GLC analysis were obtained (via the rubber sealed port) at time zero, 1 min post HCl addition, and then at 15, 30, 60, 120, 180, 300 and 360 min from the start of the hydrogenation and revealed a 90±2 % reduction of oleic acid methyl ester after 2 hours.

The procedure used for the fatty acid tritium-hydrogenation, employing a large excess of non-radioactive (cold) $NaBH_4$ chaser, was recommended by Dr R.L. Baxter (Dept. of Chemistry, University of Edinburgh). It was claimed that this cold tracer chase technique would increase tritium incorporation into the monoene. The incorporation of tritium into *cis* vaccenic acid methyl ester was 2.8 %. This gave insufficient material for the biological experiments. The extent of incorporation could be predicted when the amount by which the tritiated sodium borohydride had been diluted by the cold material was taken into consideration. Assessment of losses revealed that most of the tritium (~97 %) was present in the acidified aqueous phase. The large excess of cold $NaBH_4$ chaser was believed to have decreased the efficiency of the ^3H incorporation.

Owing to the large diluting effects of the cold $NaBH_4$, the synthesis was scaled down. Small scale two hour hydrogenations were carried out in thick glass walled vessels (1 ml, MFX, Camlab, Cambridge, Cambridgeshire, U.K.). As in the larger scale hydrogenations, ~3 mg NaB^3H_4 were decanted into the oven dried vessel followed by *cis* vaccenic methyl ester (33.5 µl), carbon (1.5 mg in 150 µl dry ethanol) and platinic chloride (0.3 mg in 45 µl dry ethanol). The vessel was then tightly closed. HCl (0.6 M) was added slowly to the system via a 23 g Microlance® needle and 1 ml Plastipak® syringe (Becton Dickinson, Dublin, Ireland) to create the tritium atmosphere. The synthesis mixture was sonicated for 2 h (to ensure adequate mixing) and the reaction mixture worked up. Results of the smaller scale hydrogenations again showed low tritium incorporation with only 5.5 and 3.7 % from two syntheses, but this was adequate for the biological experiments.

2.6.2 Purification

The reduction mixture, containing both saturated and monounsaturated fatty acid methyl esters, was purified using $AgNO_3$-impregnated Florisil™ (acid washed) column chromatography (Willner, 1961). Acid washed Florisil™ was prepared as described by Carroll (1961) and $AgNO_3$ impregnation by the method of Anderson and Hollenbach (1965).

For the large scale separations (i.e. ~0.9 g fatty acid methyl esters) a 70 cm column (i.d. 0.8 cm) was employed. The smaller scale purifications used a 5 cm column. Both columns were protected from light using aluminium foil. The $AgNO_3$-impregnated Florisil™ was poured into the columns as a slurry in hexane-diethyl ether (199:1, v/v). Elution of 92 % of the stearate methyl ester was achieved with 180 ml and 30 ml hexane-diethyl ether (199:1, v/v) from the 70 cm and 5 cm columns respectively with retention of the methyl ester of 18:1 n-9.

2.7 Analyses of [1-^{14}C] linoleic and [11,12-^{3}H] stearic acids and metabolites *in vivo*

2.7.1 Plasma

Plasma aliquots (40 µl) were counted in duplicate with 10 ml Optiphase 'safe' scintillant (F.S.A. Laboratory Supplies, Loughborough, Leicestershire, U.K.) for ^{14}C and ^{3}H labels on a Model 1209 Rackbeta scintillation counter. The Three Over Two dual counting method (Pharmacia Wallac Ltd., Uppsala, Sweden) with external standardisation was used. This facility employs three fixed counting windows and mathematically adjusts sample counts for the extent of quenching. This method provided good counting conditions for both ^{14}C and ^{3}H and after correction the degree of ^{14}C spill into the ^{3}H channel was low (0.2±0.02 %).

Individual classes of plasma lipids were extracted and separated as described in 2.5.4. and 2.5.2 respectively. They were then scraped from the TLC plates and counted with scintillant (0.125 % butyl-PBD in toluene) using the dual counting facility with external standardisation.

2.7.2 Liver microsomal phospholipid fatty acids

Phospholipids were isolated and transmethylated from liver microsomes as described in Section 2.5.2. The FAME extract was divided into three aliquots. Twenty percent of the extract was analysed by GLC (as described in Section 2.5.1). The remaining extract (80 %) was divided into two equal parts and separated by argentation chromatography. One aliquot was separated using the TLC method for the Δ6-desaturase assay. Bands of silica containing saturates and monoenes (together), dienes, trienes, tetraenes and pentaenes were scraped from the plates into separate vials and counted with scintillant (0.125 % butyl-PBD in toluene) using the dual label facility. FAME from the remaining aliquot were isolated using the TLC separation technique for Δ9-desaturase. Bands of silica containing saturated and monoene fatty

acids were separately scraped from the TLC plates and counted with scintillant. All separations were carried out within the same day following extraction.

Typical R*f* values for the Δ6 separation were as follows:-

Saturates and monoenes	0.98
Dienes	0.90
Trienes	0.81
Tetraenes	0.75
Pentaenes	0.52
Hexaenes	0.30

Typical R*f* values for the Δ9 separation were as follows:-

Saturates	0.46
Monoenes	0.20

2.7.3 Adipose tissue triacylglycerol fatty acids

The triacylglycerol fraction from freeze-dried adipose tissue was extracted and transmethylated as in Sections 2.5.6 and 2.5.2, respectively. The FAME extract was then divided into three aliquots. Twenty percent of the extract was analysed by GLC (Section 2.5.1) and the remaining extract (80 %) was divided into two equal parts and separated by argentation chromatography as described in Section 2.7.2.

2.8 Statistical analyses

Statistical analyses were conducted using MINITAB version (CLE.COM Ltd., Birmingham, U.K.). Biochemical measurements were normally distributed and the statistical protocol was one- or two- way analysis of variance (ANOVA), followed by unpaired Student's t-test. For unbalanced data (with missing values or unequal group sizes) that were unable to be analysed by two way ANOVA, the MINITAB general

linear model (GLM) was used. All results are presented as mean ± one standard deviation. Statistical significance was accepted when $p<0.05$ was achieved.

2.9 Materials

All chemical supplies were of at least analytical grade. Other than the reagents specified below or detailed in the text, all were purchased from Sigma Chemical Co., Ltd., Poole, Dorset, U.K.

Aldrich Chemical Co., Ltd., Gillingham, Dorset, U.K.

Sodium methoxide

BDH (Chemicals) Ltd., Poole, Dorset, U.K.

Carbon (decolorising purified, Norit GSX), copper sulphate ($CuSO_4.5H_2O$), diethyl ether, disodium EDTA, ethanol (dry), formic acid, hydrochloric acid (concentrated), magnesium chloride ($MgCl_2.6H_2O$), methanol (sodium dried), potassium chloride, potassium hydroxide, potassium phosphate (KH_2PO_4), silver nitrate, sodium carbonate, sodium chloride, sodium-potassium tartrate, sucrose, toluene (Aristar®)

Rathburn Chemicals Ltd., Walkerburn, U.K.

Acetic acid, chloroform, diethyl ether, heptane, hexane, methanol, 2-propanol, toluene

Chapter 3

Methodological aspects in the determination of the activity of linoleic acid Δ6-desaturase

3.1 Introduction

Most of our current knowledge on the Δ6-desaturation of 18:2 n-6 is based on studies using a radio-labelled *in vitro* activity assay in liver microsomal preparations (De Gómez Dumm *et al.*, 1979; Garg *et al.*, 1988a; Ulmann *et al.*, 1991a). Within this system, the study of lipid metabolism is a complex process in which the lipid under investigation must be dispersed in such a form that it is metabolised effectively. For Δ 6-desaturase in liver microsomes a supply of Mg^{2+}, ATP and CoASH is required for the activation of [1-^{14}C]18:2 n-6 (usually albumin bound) by acyl-CoA synthetase to produce [1-^{14}C]linoleoyl-CoA (Brenner and Catalá, 1971). Activated 18:2 n-6 is subsequently oxidatively desaturated by the Δ6-desaturase enzyme system in the presence of O_2 and NADH to produce [1-^{14}C]γ-linolenyl-CoA, which is then isolated and quantified.

Our initial results yielded low enzyme activities at the higher microsomal protein concentrations (2-5 mg) which are commonly used in some of the assay systems currently in the literature (Garg *et al.*, 1988a). This merited further investigation. This work reports the results of a study of factors influencing the 18:2 n-6 Δ6-desaturase assay, in particular the role of NaF and the importance of substrate to microsomal protein ratio.

3.2 Methods

Chow fed rats (200-300 g) were sacrificed and liver microsomes prepared as described in Section 2.3.1. Microsomal pellets were resuspended in homogenisation buffer, frozen at -20°C and analysed within four weeks. Microsomal protein concentration was analysed as outlined in Section 2.4.5. The standard incubation

mixture for Δ6-desaturase estimation consisted of the co-factors detailed in Section 2.4.8.

Before embarking on our studies on the Δ6-desaturase assay, a thorough appraisal of factors that affect the blank value was made in the system. Linoleic acid is susceptible to oxidation. Products originating from this process have a reduced mobility during argentation chromatography and may therefore coincide with the 18:3 n-6 band, resulting in a high blank. We therefore examined whether flushing tubes with argon and the addition of BHT (0.005 % or 0.05 % (w/v)) as an anti-oxidant to all solvents during the extraction, derivitisation, and chromatographic procedures could reduce the blank. The results demonstrated that blanks (i.e. no microsome addition) could be maintained at the lowest and most reproducible level (0.30±0.03 % of total counts) when samples were argon flushed and all solvents contained 0.005 % (w/v) BHT.

The influence of microsomal protein (0.25-5.0 mg) on Δ6-desaturase activity using a fixed amount of [1-^{14}C]18:2 n-6 (200 nmol) as substrate was investigated with a 20 min incubation and the cofactors as described in Section 2.4.8.

The effect of NaF (to inhibit ATPase) was investigated with 0.25-1.5 mg microsomal protein. Incubations were carried out in the presence or absence of 45 mM NaF, with 200 nmol [1-^{14}C]18:2 n-6 and a 20 min incubation. ATP concentrations were measured in duplicate samples as described in Section 2.4.7.

The use of an ATP re-generating system was assessed (without NaF) with the standard incubation medium supplemented with creatine phosphate (20 mM) and creatine kinase (80 U/incubation (Reddy, 1989)). ATP and creatine phosphate measurements were made as described in Section 2.4.7.

The effect of substrate concentration was investigated over a range of 40-200 nmol of [1-^{14}C]18:2 n-6 with the standard amount of 0.5 mg microsomal protein. The effect of maintaining a constant ratio of substrate to microsomal protein was also

investigated utilising the standard incubation conditions with a microsomal protein range of 0.25-1.8 mg and [1-^{14}C]18:2 n-6 range of 100-800 nmol.

In order to examine whether the lower activities were due to a relatively non-specific binding effect on the substrate we also studied the effect of bovine serum albumin. Albumin at two levels (0.5 mg and 1.5 mg, Fraction V, essentially fatty acid free) with a fixed amount of [1-^{14}C]18:2 n-6 (200 nmol) was added to microsomal protein (0.5 mg) under the standard incubation conditions. The effect of controlling the 18:2 n-6 to albumin ratio was also studied by increasing the *total* 18:2 n-6 concentration to 400 and 800 nmol in the incubations that contained 0.5 and 1.5 mg albumin, respectively.

Finally we decided to examine the influence of anaesthesia (sodium pentobarbital, 60 mg/kg, Sagatal®, Rhône Mérieux Ltd., Dublin, Ireland) in a small number of rats (three per group). The effect of freezing (for one hour, one week, two weeks and four weeks, at -20°C) on Δ6-desaturase activity was also examined in liver microsomes from four rats.

3.3 Results

The amount of [1-^{14}C]18:2 n-6 converted over a broad range of microsomal protein concentrations was maximal at *ca.* 0.75 mg microsomal protein, with linearity to this level (Fig. 3.1). Thereafter a steady decline was observed in 18:2 n-6 conversion with the lowest activity at 5 mg microsomal protein.

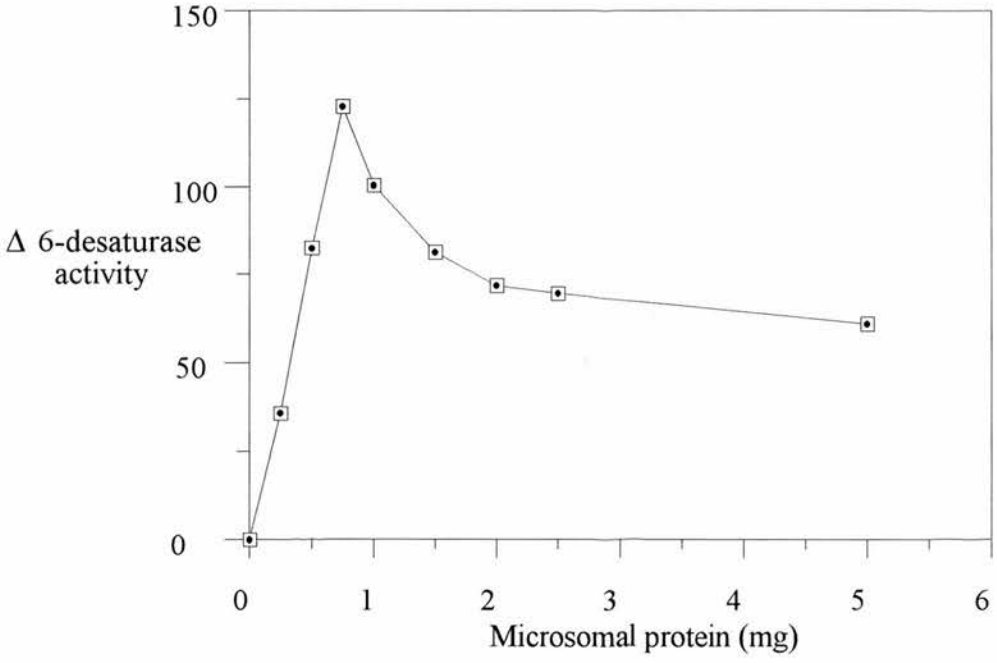


Figure 3.1 *Effect of microsomal protein concentration on Δ6-desaturase activity (pmol/min) using a fixed amount of [1-^{14}C]18:2 n-6 (200 nmol). Each point represents the mean of duplicate analyses. Pattern is typical of three separate experiments. Values of activity are lower than those presented in Figures 3.2, 3.3 and 3.4 and relate to the variability in activity of liver microsomal Δ6-desaturase from different batches of rats.*

NaF tended to increase the activity of Δ6-desaturase using <0.5 mg microsomal protein by about 10 %; however, this result was not significant ($p>0.05$, Table 3.1). The effect of NaF at higher microsomal protein concentrations (i.e. >0.5 mg) was more marked with a significantly lower (not higher) Δ6-desaturase activity. Higher ATP concentrations were maintained during the 20 min incubations with NaF. Some 90 % of the added ATP remained at the end of the 20 min incubation with NaF when a small amount of microsomal protein was used (0.33 mg). Less ATP was found in two other experiments when 0.62 mg and 1.1 mg microsomes were used: 75 % and 65 %, respectively. In the absence of NaF the respective figures in these experiments were 78 %, 59 %, and 38 % of ATP remaining.

Table 3.1 *Effect of inhibition of endogenous ATPases by NaF and re-generation of ATP on liver microsomal Δ6-desaturase activity*

Incubation conditions	n	Activity	p value
Control		100 %	
+ 45 mM NaF	3	110±11 %	n.s.
+ 45 mM NaF #	4	82±7 %	0.005
+ ATP re-generation system	3	94±7 %	n.s.

Results expressed as percentage of relevant control (Mean±SD). # Addition of high microsomal protein (>0.5 mg). n.s. Not significant.

The use of an ATP re-generating system maintained ATP concentrations at a constant 4 mM throughout the incubation period (measured at 5, 10, 15 and 20 min) and was coupled with a concomitant decline in creatine phosphate levels (data not shown). The maintenance of ATP concentration had no effect on Δ6-desaturase activity (Table 3.1).

Under standard incubation conditions (i.e. 0.5 mg microsomal protein in a medium devoid of both NaF and the ATP re-generating system), the activity of Δ6-desaturase activity was constant up to 20 min incubation (Fig. 3.2).

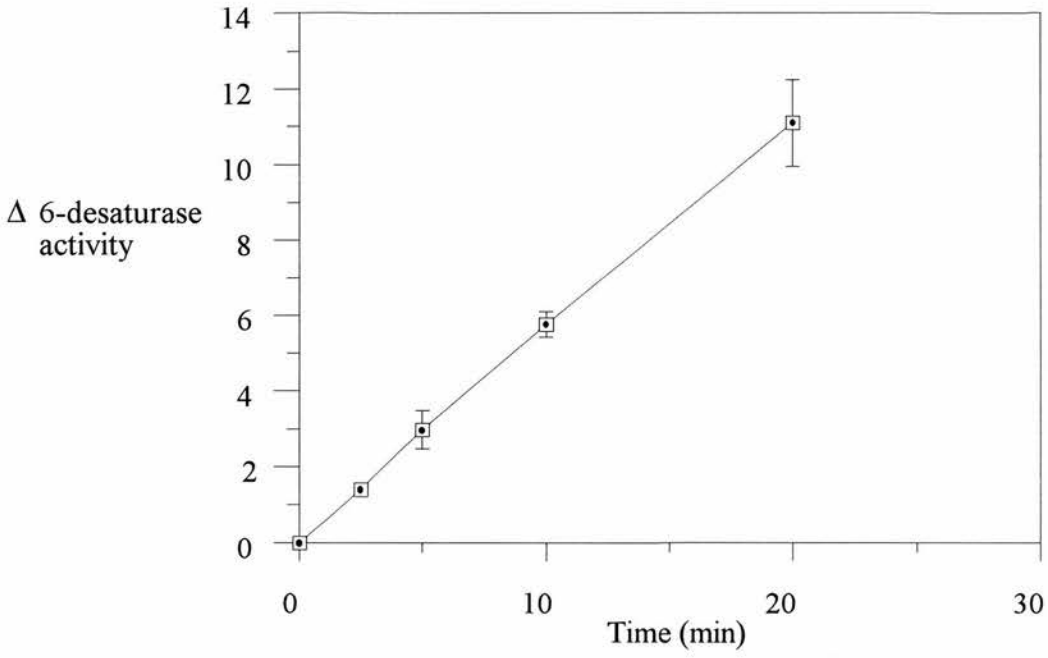


Figure 3.2 *Conversion of [1-^{14}C]18:2 n-6 (nmol/mg) by liver microsomal Δ6-desaturase in relation to time of incubation using a fixed amount of [1-^{14}C]18:2 n-6 (200 nmol). Each point represents the mean of triplicate analyses. Results expressed as Mean±SD. Pattern is typical of three separate experiments.*

The effect of substrate concentration on Δ6-desaturase activity, analysed over a 40-200 nmol range, demonstrated an S-shaped relationship (Fig. 3.3). Maximal Δ6-desaturase activity with 0.5 mg microsomal protein was obtained between 150 and 200 nmol 18:2 n-6. Activities for Δ6-desaturase assessed using 2 mg microsomal protein were considerably lower. They did not reach their peak activity at the substrate concentrations used.

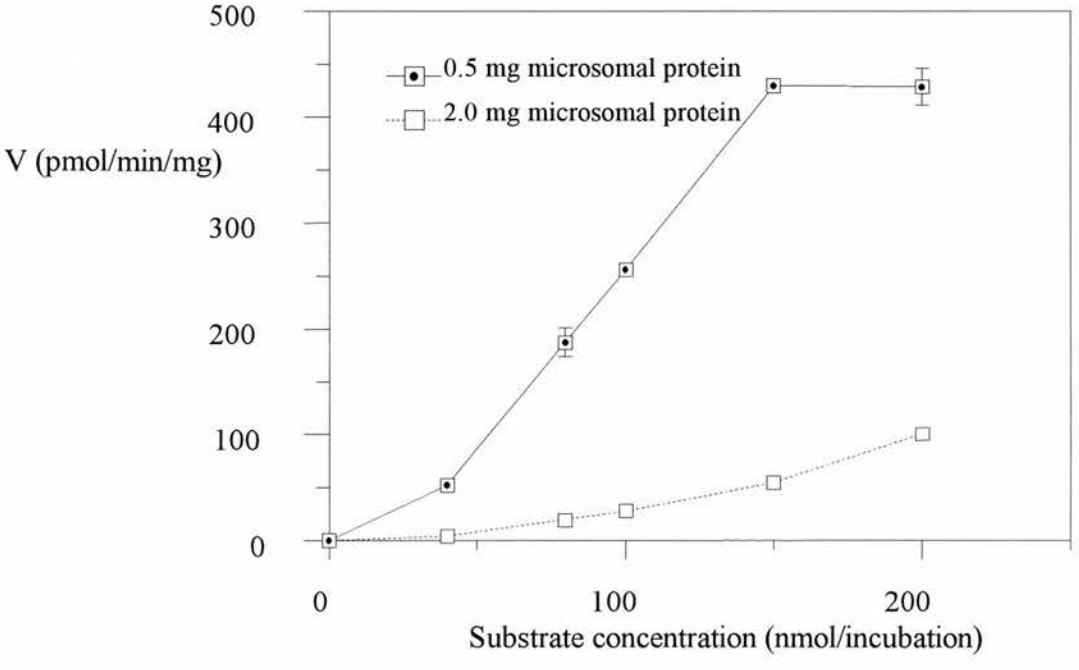


Figure 3.3 *Effect of substrate concentration on Δ6-desaturase activity. The conversion of [1-^{14}C]18:2 n-6 by liver microsomes (0.5 and 2.0 mg protein) was examined in relation to the amount of substrate added (40-200 nmol per test). No attempt was made to control the substrate to protein ratio in this experiment. Each point represents the mean of triplicate analyses. Results expressed as Mean±SD. Pattern is typical of two separate experiments for the analysis with 0.5 mg microsomal protein.*

Albumin addition (0.5 mg) caused a significant (17 %) decrease in Δ6-desaturase activity ($p<0.02$). Furthermore, 1.5 mg albumin reduced the activity by 60 % ($p<0.002$, Table 3.2). When 0.5 mg albumin was added simultaneously with 200 nmol extra 18:2 n-6 (final 400 nmol, to maintain a similar substrate-albumin ratio) no change in Δ6-desaturase activity was observed. However, 1.5 mg albumin addition showed a 20 % decrease in Δ6-desaturase activity despite additional 600 nmol 18:2 n-6 (final 800 nmol).

Table 3.2 *Effect of the addition of bovine serum albumin on liver microsomal Δ6-desaturase activity*

Incubation conditions	n	Activity	p value
Control		100 %	
+ Albumin (0.5 mg)	3	83±3 %	0.015
+ Albumin (1.5 mg)	3	40±2 %	0.002
+ Albumin (0.5 mg) plus 18:2 n-6 §	2	103 %	-
+ Albumin (1.5 mg) plus 18:2 n-6 ‡	2	80 %	-

Results expressed as percentage of control (Mean±SD). § Control incubation with additional albumin (0.5 mg) and extra 18:2 n-6 (+ 200 nmol, thus final 400 nmol) to provide approximate fatty acid/albumin molar ratio of 23:1. ‡ Control incubation with additional albumin (1.5 mg) and extra 18:2 n-6 (+ 600 nmol, thus final 800 nmol) to provide approximate fatty acid/albumin ratio of 24:1. - Not statistically feasible.

When the ratio of [1-^{14}C]18:2 n-6 to *microsomal* protein concentration was kept constant, a linear relationship between Δ6-desaturation and microsomal protein occurred (Fig. 3.4).

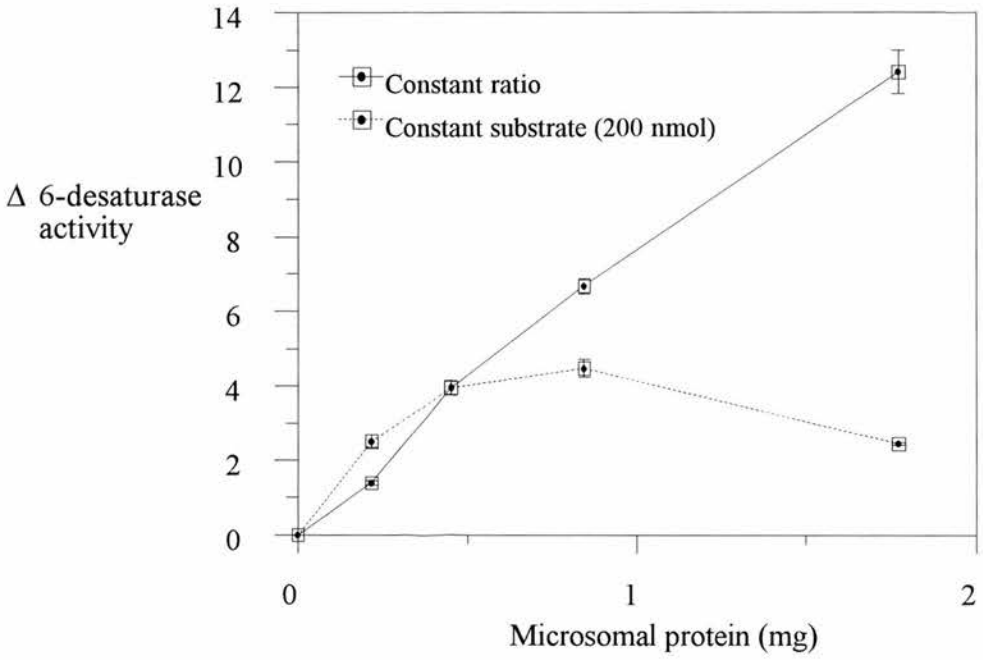


Figure 3.4 *Conversion of [1-^{14}C]18:2 n-6 by Δ6-desaturase in liver microsomes in relation to the amount of microsomal protein and substrate. Note linear conversion with the fixed substrate-protein ratio, but not when the substrate was maintained at 200 nmol with increasing microsomal protein. Results expressed as nmol converted per 20 min incubation (Mean±SD). Pattern is typical of two separate experiments.*

In contrast, when the substrate concentration was kept constant the expected decline in Δ6-desaturase activity was evident at the higher microsomal protein concentrations.

Neither anaesthesia nor storage affected liver microsomal Δ6-desaturase activity using the standard assay conditions (Table 3.3).

Table 3.3 *The effect of anaesthesia and storage on microsomal Δ6-desaturase activity*

Preparation/incubation	Activity	p value
Control microsomes (stunned rats, n=3)	100 %	
Microsomes from anaesthetised rats (n=3 rats) §	99.6±16.4 %	n.s.
Control microsomes (fresh, n=4 livers)	100 %	
Frozen one hour (n=4 livers) †	103.7±13.5 %	n.s.
Frozen one week	103.2±17.6 %	n.s.
Frozen two weeks	113.5±11.2 %	n.s.
Frozen four weeks	105.6±15.6 %	n.s.

§ Rats were anaesthetised with Sagatal® (60 mg/kg body weight, intraperitoneally); † All frozen microsomal preparations were stored at -20°C and thawed on ice. All results are expressed as percentage of control. Standard Δ6-desaturase incubation conditions were used. n.s. Non significant.

3.4 Discussion

Although the activity of Δ6-desaturase is highest in mammalian liver compared to other organs, the actual rate of conversion for 18:2 n-6 is low. Thus, large amounts of microsomes (up to 5 mg protein) are normally used to maximise substrate conversion. This study shows that using high microsomal protein concentrations to measure Δ6-desaturase *in vitro* leads to a serious underestimation of its activity. This effect can be attributed to the non-specific binding of [1-^{14}C]18:2 n-6 to microsomal protein since bovine serum albumin, which effectively binds non-esterified fatty acids (Spector *et al.*, 1969), also resulted in reduced Δ6-desaturase activity. This effect, however, does not manifest itself if the 18:2 n-6 to protein ratio is kept constant. On the other hand, Δ6-desaturase activity was not influenced by the presence of endogenous ATPases, competing for ATP and thereby preventing the activation of the non-esterified acid by microsomal acyl-CoA synthetase. Thus, addition of NaF which inhibits ATPase did not

result in a significant increase in Δ6-desaturase activity. The observed reduction in Δ6-desaturase activity is perhaps due to the formation of insoluble MgF_2 and hence a reduction in free Mg^{2+} concentration.

The results therefore demonstrate that fatty acid-protein interactions in this system create a prominent and important effect. Interestingly, Bar-Tana *et al.* (1971) have shown a 70 % inhibition of palmitoyl-CoA synthetase by albumin addition. Inconsistent with this, are the findings of Cook (1978) who demonstrated that albumin additions had no effect in a brain microsomal Δ6-desaturase system. A closer examination of both systems reveals the inhibitory effect of albumin on palmitoyl-CoA synthetase could be abolished by the addition of the surface active compound Triton X-100, while the brain microsomal Δ6-desaturase assay used 25 μM Triton WR 1332. As the future work was to include an assessment of Δ6-desaturase in dietary cholesterol enriched microsomal membranes we did not wish to include a detergent.

Alternatively, lipid-protein interactions may be overcome by controlling the substrate-protein ratio as performed in this study. It is possible that some of the fatty acids occupy non-specific binding sites on the microsomal protein. However, an assessment of fatty acid binding properties of microsomal protein was not carried out in a formal manner. But the conclusion seems justified in view of similar results with additional albumin.

The method described here uses argentation-TLC combined with scintillation counting to achieve the best sensitivity. The use of gas chromatography with radiochemical detection is frequently used for Δ6-desaturase evaluation owing to the greater specificity. Nevertheless, the present results pertain to incubation conditions and therefore are still appropriate for Δ6-desaturase methods using gas-liquid chromatography.

Sodium pentobarbital anaesthesia had no significant effect on the activity of Δ6-desaturase compared with the quicker form of killing (stunning with cervical

dislocation). Therefore, results from studies using either means of sacrifice are comparable.

Several authors have determined liver microsomal Δ6-desaturase activity after freezing (Eck *et al* ., 1979; Faas and Carter, 1980; Shimp *et al*., 1982; De Shrijver and Privett, 1983; Biagi *et al*., 1991; Ruiz-Gutierrez and Muriana, 1992). Yet, there is little information on the effect of prolonged freezing. Faas and Carter (1980) have documented that Δ6-desaturase activity is stable for up to two weeks when frozen in a concentrated form (15 mg microsomal protein/ml buffer). This study showed no significant storage effects on Δ6-desaturase activity for up to four weeks at -20°C (microsomal protein concentration ~40 mg/ml). This information is useful since it means that the sacrifice, liver microsome preparation and Δ6-desaturase activity analysis do not have to be performed within the same day.

In conclusion, Δ6-desaturase activity in rat liver microsomes can be measured without the addition of NaF or an ATP re-generating system. The effects of substrate interaction with protein can be marked at high microsomal protein levels and must be realised. Under the conditions described protein binding to the fatty acid substrate is not a serious problem. The revised method using 0.5 mg microsomal protein, 200 nmol substrate and 20 min incubation assesses adequately the activity of Δ6-desaturase *in vitro.*

Chapter 4

Linoleic acid metabolism in the rat
The role of dietary cholesterol

4.1 Introduction

Epidemiological studies, examining risk markers involved in the pathogenesis of CHD in man, have frequently implicated serum cholesterol as having an important and strong association (Keys *et al.*, 1981; Kannel and Gordon, 1982; Martin *et al.*, 1986; Law and Wald, 1994). Yet, such studies are constrained by the small number of tissues available for in-depth study and so limited information exists on tissues that perform large scale EFA metabolism (e.g. liver). Plasma and adipose tissue analysis have offered some information in human studies (Wood *et al.*, 1984; Riemersma *et al.*, 1986) but do not answer more fundamental questions on the interaction between dietary constituents and lipid metabolism.

Although cholesterol in many situations has been regarded as an *enfant terrible* by the nutritional media, it is an essential molecule performing a wide range of key biological functions that include its constituent role as a membrane fluidity regulator. The structure of cholesterol is amphipathic in nature and it is well suited to mesh within lipid bilayers (Yeagle, 1985; Brenner, 1990). Increased cholesterol concentrations in cell membranes, by way of interacting with phospholipid esterified fatty acids, will decrease membrane 'fluidity' (Yeagle, 1985; Brenner, 1990) and alter enzyme activities (Garda and Brenner, 1985; Castuma and Brenner, 1986). Membrane bound enzymes, such as glucose-6-phosphatase, are inhibited in cholesterol enriched intact microsomes but not after microsomal disruption using detergents (Garda and Brenner, 1985). Yet, NADH-ferricyanide reductase activity, a measure of NADH-cytochrome b_5 reductase, is unaffected by membrane cholesterol enrichment (Garda and Brenner, 1985). The multi-component enzyme adenylate cyclase, which relies upon 'collision coupling' between its subunits, is activated by reducing membrane cholesterol levels (Salesse *et al.*, 1982; Needham *et al.*, 1985). Multi-component

microsomal enzyme systems such as the desaturases are believed to be regulated by the fluidity of their surrounding membrane (Brenner, 1990). These enzymes themselves also influence membrane fatty acid composition.

The aim of this investigation is to assess Δ6-desaturase activity in liver microsomes, using our newly optimised method that does not use detergents, from cholesterol supplemented rats and to compare this with changes in the index for Δ6-desaturase as derived from fatty acid composition of microsomal phospholipids and adipose tissue triacylglycerols.

4.2 Methods

Male Sprague Dawley rats (n=16, ~100 g) were randomised into two groups and fed either the basal semi-synthetic diet (Section 2.2) or the same diet enriched with 2 % (w/w) cholesterol. Control and experimental diets were provided *ad libitum* for 27 days. Animals were housed as described (Section 2.1), examined regularly and weighed weekly to ensure they were in good health for the duration of the experiment. Rats of both groups were sacrificed under standardised conditions (Section 2.3.1) and in the fed state to control for diurnal variations and to maximise Δ6-desaturase activity. Tissues were collected over two separate days to facilitate rapid sample processing (four control and four experimental rats per day). Microsomes were prepared (Section 2.3.1) and assessed for protein content and mitochondrial contamination (Sections 2.4.5 and 2.4.6, respectively). Δ6-desaturase activity was measured under conditions described in Section 2.4.8. Microsomal phospholipid fatty acid composition and microsomal cholesterol content were analysed as in Sections 2.5.2 and 2.5.7, respectively. Adipose tissue triacylglycerol fatty acid composition was analysed as described (Section 2.5.6).

4.3 Results

4.3.1 General animal characteristics

The animals remained healthy throughout the duration of the study. Growth rates of the two groups of rats were in close agreement (Fig. 4.1).

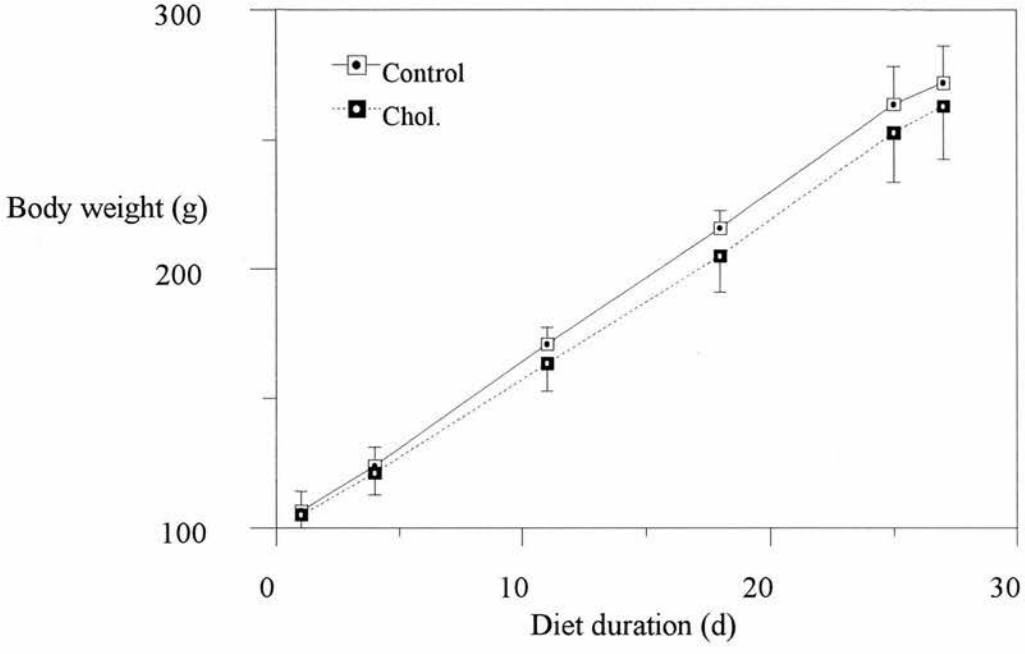


Figure 4.1 *Total body weights during the experiment of rats fed a control or cholesterol enriched diet. Values expressed as Mean±SD.*

No significant effect of cholesterol supplementation on body weight, liver weight and heart weight was evident at the end of the study (all $p>0.05$, Table 4.1). Of interest, however, was a significantly elevated liver weight to body weight ratio in the cholesterol supplemented group ($p=0.0015$, Table 4.1). A similar relationship was not apparent for heart weight.

Data for the remainder of the study consists of seven animals in the control group and eight animals in the cholesterol supplemented group as one liver homogenate was lost through a broken tissue grinder.

Table 4.1 *Body weights, liver weight and heart weight of rats fed a control or cholesterol enriched diet*

Measurement	Control n=8	Chol. n=8
Weight (g)		
Body		
Initial	107±8	105±8
Final	272±14	263±20
Liver	12.4±1.4	13.7±1.4
Heart	1.06±0.13	1.00±0.09
Relative weight (%)		
Liver/body weight	4.5±0.4	5.2±0.3**
Heart/body weight	0.39±0.03	0.38±0.02

Values expressed as Mean±SD. ** $p<0.01$ *vs.* control.

4.3.2 Microsomal phospholipid and cholesterol content

As expected cholesterol feeding increased the total cholesterol content of liver microsomes by 56 % ($p=0.0005$, Table 4.2). Cholesterol supplementation had no effect on the phospholipid content of liver microsomes expressed per mg protein. Nevertheless the resultant effect was to increase the cholesterol to phospholipid ratio by 58 % in the cholesterol fed animals ($p=0.0003$).

Table 4.2 *Microsomal cholesterol and phospholipid contents and Δ6-desaturase and succinate dehydrogenase activities of rats fed a control or cholesterol enriched diet*

Measurement	Control n=7	Chol. n=8
Cholesterol ξ	49±7	77±15***
Phospholipid ξ	425±42	405±30
Cholesterol/phospholipid ratio ψ	0.12±0.01	0.19±0.04***
Δ6-desaturase activity ζ	409±91	517±73*
Succinate dehydrogenase activity ¥	1.7±0.9	1.7±0.4

Values expressed as Mean±SD. * $p<0.05$, *** $p<0.001$ *vs.* control. ξ nmol/mg protein, ψ mol/mol, ζ pmol/min/mg, ¥ nmol/min/mg protein.

4.3.3 Microsomal Δ6-desaturase activity

The 27 day cholesterol supplementation regimen elevated liver microsomal Δ6-desaturase activity by 26 % compared with control fed animals ($p=0.025$, Table 4.2). No significant correlation existed between Δ6-desaturase activity and cholesterol/ phospholipid ratio or microsomal total cholesterol content. Microsomal succinate dehydrogenase activity was similar between the two groups when expressed per mg microsomal protein. Represented as a percentage of total homogenate activity, the mitochondrial contamination was 0.05±0.02 % for the control microsomes compared with 0.17±0.09 % for microsomes from cholesterol fed rats ($p<0.05$).

4.3.4 Microsomal phospholipid fatty acid composition

Cholesterol feeding induced widespread significant modifications to microsomal fatty acid composition (Table 4.3). Overall, the cholesterol supplement increased the percentage of MUFA by 50 % (mainly 18:1, but also small significant increases occurred for 16:1 and 20:1) with a concomitant decline in SFA.

No change in the total percentage of n-6 fatty acids was detected but a distinct shift in the distribution therein was apparent. Microsomal phospholipids from rats fed cholesterol were enriched with 18:2, 18:3 (0.14±0.04 *vs.* 0.08±0.02 %) and 20:3 compared with control. These increases were accompanied by significantly decreased amounts of both 20:4 and 22:4, though 22:5 was not different.

Table 4.3 *Liver microsomal total phospholipid fatty acid composition of rats fed a control or cholesterol enriched diet*

Fatty acid species	Control n=7	Chol. n=8
SFA		
14:0	0.1±0.0	0.2±0.1**
16:0	15.3±0.4	14.1±1.0**
18:0	30.9±0.5	27.8±1.4***
MUFA		
16:1	0.6±0.0	1.1±0.3**
18:1	9.1±0.4	13.6±0.8***
20:1	0.1±0.0	0.3±0.1***
PUFA (n-6)		
18:2	11.0±0.6	13.0±1.5**
20:2	0.1±0.0	0.2±0.0**
18:3 #	0.1±0.0	0.1±0.0**
20:3	1.8±0.2	2.4±0.2***
20:4	24.1±0.5	21.4±1.6***
22:4	0.3±0.0	0.2±0.0***
22:5	1.1±0.3	0.8±0.4
PUFA (n-3)		
20:5	0.1±0.0	0.2±0.0*
22:5	0.5±0.1	0.4±0.1*
22:6	4.3±0.1	3.5±0.2***
PUFA (n-9)		
20:3	0.4±0.1	0.7±0.1***
Σ SFA	46.3±0.4	42.1±0.9***
Σ MUFA	9.9±0.4	15.0±1.0***
Σ n-6	38.4±0.2	38.1±0.6
Σ n-3	5.0±0.1	4.1±0.2***
DBI	171±2	166±5*

Values expressed as % (w/w) of total fatty acids (Mean±SD). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs.* control. Isomeric forms of 18:1 not elucidated. # Unresolved peak contained unspecified amounts of 20:0. DBI double bond index.

The alterations in n-6 fatty acid composition were accompanied by reductions in the relative amounts of both 22:5 n-3 and 22:6 n-3 culminating in a significantly reduced double bond index (DBI). The lower DBI was associated with a 75 % increase in 20:3 n-9 in cholesterol fed rats.

4.3.5 Adipose tissue triacylglycerol fatty acid composition

The dietary cholesterol induced modifications to adipose tissue triacylglycerol fatty acid composition were considerably more limited when compared to those found in microsomal membrane phospholipids (Table 4.4).

Table 4.4 *Adipose tissue triacylglycerol fatty acid composition of rats fed a control or cholesterol enriched diet*

Fatty acid species	Control (n=7)	Chol. (n=8)
SFA		
14:0	2.5±0.2	2.3±0.2
16:0	21.8±0.7	21.3±0.6
17:0	0.7±0.1	0.8±0.2
18:0	9.2±0.9	10.0±1.2
20:0 #	0.1±0.0	0.1±0.0
MUFA		
16:1	5.7±0.7	5.1±0.9
18:1	45.4±0.9	46.6±0.9*
20:1	1.0±0.1	1.1±0.1
PUFA(n-6)		
18:2	12.1±1.0	11.3±0.4
20:3	n.d.	n.d.
20:4	0.1±0.0	0.1±0.0
PUFA (n-3)		
18:3	0.4±0.1	0.3±0.1
Σ SFA	34.3±1.5	34.4±0.8
Σ MUFA	52.1±0.8	52.8±1.2
Σ n-6	12.2±1.0	11.4±0.4
Σ n-3	0.4±0.1	0.4±0.1
Unknown	1.0±0.2	1.0±0.1
DBI	78±2	77±2

Values expressed as % (w/w) of total fatty acids (Mean±SD). * $p<0.05$ *vs.* control. # Unresolved peak contained unspecified amounts of 18:3 n-6. n.d. Not detectable.

The only fatty acid to alter, as a result of the cholesterol feeding, was 18:1 (mainly 18:1 n-9) which was significantly increased ($p=0.023$). This modification was in the same direction as that detected for 18:1 in the microsomal phospholipid fraction. Conversely, the relative amount of adipose tissue 18:2 n-6 showed an opposite trend ($p=0.08$); increased in microsomal phospholipids but reduced in adipose tissue. These modest changes in adipose tissue fatty acid composition led to a significant elevation in the 18:1/18:2 n-6 ratio from 3.8±0.4 to 4.1±0.2 ($p=0.04$).

As only modest changes to adipose tissue fatty acid composition were observed, ratios pertinent to the metabolism of 18:2 n-6 were not modified by cholesterol supplementation.

The relative amount of 18:2 n-6 in adipose tissue has been regarded as a valid, long term indicator of dietary intake in men (Dayton *et al.*, 1966; Wood *et al.*, 1984). Since diet is considerably more controlled in animal studies, it is of interest to compare diet and tissue fatty acid compositions. A comparison between dietary fatty acids and mean adipose tissue fatty acid composition for the two groups is shown in Fig. 4.2.

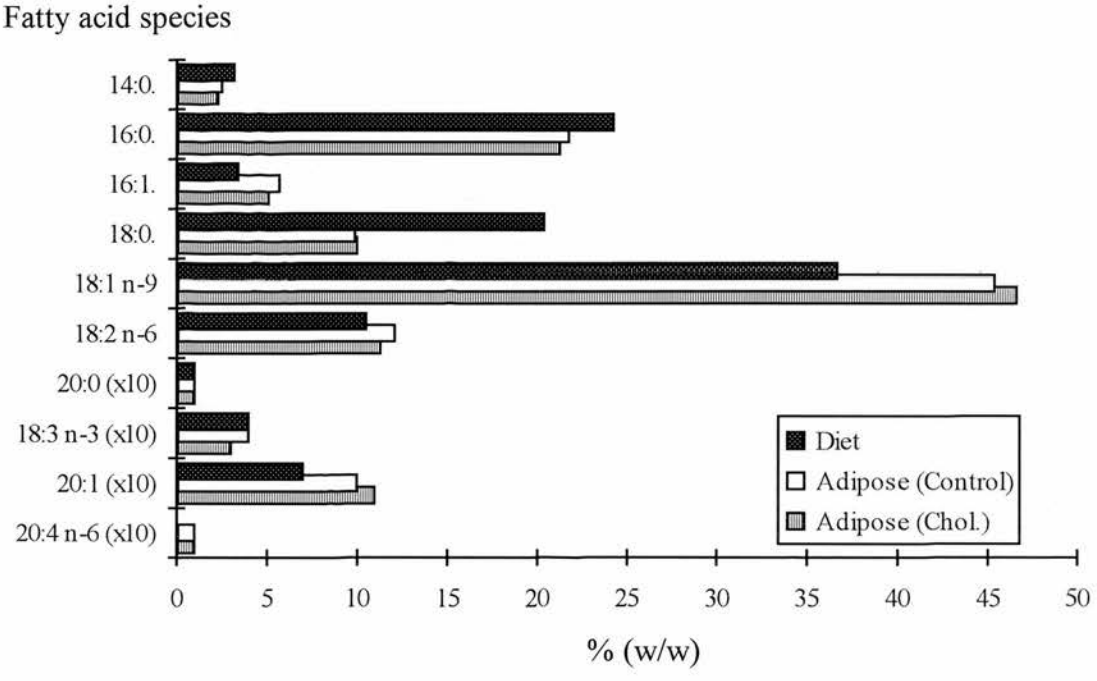


Figure 4.2 *Comparison between fatty acid composition of the diet and adipose tissue of rats fed a control or cholesterol enriched diet. Results expressed as % (w/w) of total fatty acids.*

Generally, the relative amounts of fatty acids in both adipose tissue and diet were similar with 18:1 and 16:0 forming the bulk content. An exception to this was 18:0 which was 50 % greater in the dietary fat compared to adipose tissue. Conversely, there was a 25 % increase in adipose tissue 18:1 compared to diet. Both EFA (18:2 n-6 and 18:3 n-3) in the diet reflected in their relative amounts in adipose tissue.

4.3.6 Microsomal phospholipid fatty acid ratios

It is a common approach to use a product-precursor ratio of fatty acids from a particular lipid pool as an indicator of n-6 fatty acid metabolism by the various desaturases. These indices are demonstrated for the phospholipid esterified fatty acids in Table 4.5.

Table 4.5 *Indices of desaturase activities derived from microsomal phospholipid fatty acid composition, and triene/tetraene ratios of rats fed a control or cholesterol enriched diet*

Fatty acid ratio	Control n=7	Chol. n=8
n-6		
18:3/18:2 (Δ6)	0.007±0.002	0.011±0.004*
20:4/20:3 (Δ5)	13.65±1.46	9.13±1.48***
22:5/22:4 (Δ6)	3.4±0.6	3.3±1.4
18:2 D&E	2.52±0.21	1.97±0.38**
n-9		
18:1/18:0 (Δ9)	0.294±0.015	0.491±0.051***
20:3 n-9/20:4 n-6	0.017±0.002	0.031±0.007***

Values expressed as Mean±SD. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs.* control. 18:2 D (Desaturation) & E (Elongation) refers to (18:3 n-6 + 20:2 n-6 + 20:3 n-6 + 20:4 n-6 + 22:4 n-6 + 22:5 n-6)/18:2 n-6.

The 18:3 n-6/18:2 n-6 Δ6-desaturase ratio was increased in the cholesterol fed group by 56 %. Using a combination of all desaturation and elongation products derived from 18:2 n-6, the apparent throughput from 18:2 n-6 was depressed by 22 % mainly due to decreased formation of fatty acids originating from 20:3 n-6. Indeed, the 20:4 n-6/20:3 n-6 ratio for Δ5-desaturase was also lowered by 33 % but the 22:5 n-6/22:4 n-6 Δ6-desaturase ratio was not different. The ratio derived for Δ9-desaturase (18:1/18:0) was augmented by cholesterol feeding. This was accompanied by an increased triene/tetraene ratio (20:3 n-9/20:4 n-6) that was almost doubled by the cholesterol supplement. Nevertheless, signs of an EFA deficiency were not observed in the living animal (i.e. no scaly skin or hair loss were visible throughout the experiment).

The n-3 fatty acid composition of microsomal phospholipids differed markedly compared to the n-6 series. α-Linolenic acid was not detectable and its Δ6-desaturation product, 18:4 n-3, was similarly absent. Indeed, the only n-3 fatty acids observed in the phospholipid fraction were those derived after Δ5-desaturation. This made it impossible to use a product-precursor ratio as an indicator for either Δ6-desaturase (18:4 n-3/18:3 n-3) or Δ5-desaturase (20:5 n-3/20:4 n-3) and highlights the differential metabolism undergone by these two distinct series of EFA. The Δ6-desaturase index estimated from 22:5 n-3 and 22:6 n-3 is possible though and the derived figures were 8.4±1.3 and 8.6±1.9 for control and cholesterol fed rats, respectively. This result did not achieve statistical significance which is in close agreement with that found using the respective n-6 fatty acids for the same index.

4.4 Discussion

Many studies have found that rat liver microsomal Δ6-desaturase activity is reduced as a result of cholesterol feeding (Leikin and Brenner, 1987; Garg *et al.*, 1988a; Muriana *et al.*, 1992). The results of Δ6-desaturase measurements demonstrated here contrast with this literature. However, the overall metabolism of 18:2 n-6 to 20:4 n-6 depends on the activity of both Δ6- and Δ5-desaturases as well as the relevant elongases. It is suggested from the lower fatty acid desaturation and elongation index that cholesterol feeding may have reduced the overall activity of this pathway.

The disparity between our results and those published may be due to a variety of reasons. Firstly, the method described in Chapter 3 was developed so that the maximum activity of Δ6-desaturase could be measured without the use of detergents, as cholesterol enrichment alters membrane fluidity (Yeagle, 1985; Brenner, 1990). The approach commonly used for Δ6-desaturase measurement uses very large amounts of microsomal protein with relatively small amounts of substrate (Garg *et al.*, 1988a; Chanussot *et al.*, 1989) that could be seen as sub-optimal. Notable are the results of

Leikin and Brenner (1987) who demonstrated that Δ6-desaturase activity measured with 60 μM [1-^{14}C]18:2 n-6 was slightly but non-significantly increased in cholesterol fed rats. The measurement with 15 μM [1-^{14}C]18:2 n-6, however, showed a significantly decreased activity in cholesterol supplemented rats. Thus indicating that substrate concentration is an important determinant of the result obtained. Unfortunately, these workers did not examine substrate concentrations greater than 60 μM.

The role of cholesterol in an *in vitro* system (using microsomes enriched *in vitro* with cholesterol rich liposomes) has also been shown to increase Δ6-desaturase by Garda and Brenner (1985). This is opposite to the commonly accepted effect of cholesterol on Δ6-desaturase activity but consistent with the data described in the present study. The effect on Δ9-desaturase activity in the same study *in vitro* was consistent with that shown for the Δ9-desaturase index in the present study using dietary cholesterol. It has been argued that within the situation of cholesterol membrane enrichment *in vivo*, other modifications take place such as those associated with phospholipid head group composition (Brenner, 1990). No significant changes were observed for the total amount of phospholipid present in microsomal membranes. The effect of dietary cholesterol on phospholipid sub-classes was not examined within the context of this study and requires further elucidation.

Isolation stress could have affected the results of Garg *et al.* (1988a). This group used essentially a similar feeding protocol to that described here, except the animals were isolated for the assessment of dietary intakes. Raised catecholamines, cortisol and blood pressure are all attributes of the socially deprived, stressed animal (Crawford and Law, 1958; Sanford *et al.*, 1986; Huang *et al.*, 1990). The mechanism whereby these hormones might affect Δ6-desaturase is not fully understood. However, an infusion of a very large dose of the stress hormone adrenaline (1 mg/kg body weight) has been shown to decrease Δ6-desaturase activity within two hours (De

Gómez Dumm *et al.*, 1976). Nevertheless, neither Garg *et al.* (1988a) nor this study examined stress levels in these animals.

The semi-synthetic diets given to these animals in the present study were completely devoid of fatty acids of the n-6 series other than 18:2 n-6. Thus, changes in desaturation and elongation products of 18:2 n-6 present in a particular lipid pool could relate to metabolic alterations *in vivo*. Large changes in the SFA and MUFA composition of microsomal phospholipids could have altered the n-6 and n-3 series purely because they are mathematically related when expressed as percentages. However, the SFA and MUFA components can be calculated out so that their effects are removed (Table 4.6). The cholesterol effect upon individual n-6 and n-3 fatty acids remains the same when this calculation is done. In addition, the fact that some of the n-6 fatty acids increase their percentage contribution while others decrease, indicates that changes in SFA and MUFA do not affect n-6 and n-3 fatty acids in a mathematical way. This suggests that the effect is of genuine metabolic origin.

Table 4.6 *Comparison of p values obtained from the statistical analyses of microsomal phospholipid n-6 and n-3 fatty acid contents allowing for SFA and MUFA contributions*

Fatty acid species	P value from normal calculation	P value allowing for SFA and MUFA content
PUFA (n-6)		
18:2	0.0062	0.0081
20:2	0.0071	0.0100
18:3	0.0036	0.0044
20:3	0.0001	0.0001
20:4	0.0020	0.0021
22:4	0.0008	0.0006
22:5	0.1100	0.0970
PUFA (n-3)		
20:5	0.0170	0.0210
22:5	0.0290	0.0200
22:6	<0.0001	<0.0001

On this basis one would conclude that cholesterol feeding causes large differences to occur in 18:2 n-6 metabolism. Most affected of the n-6 series was 20:3 n-6 which was increased by 33 % as a result of the cholesterol feeding. This, in combination with

elevated 18:3 n-6 could be indicative of increased Δ6-desaturase activity that is highlighted by the increased Δ6-desaturase (18:3 n-6/18:2 n-6) ratio. A packed column GLC was used to determine the fatty acid compositions and unfortunately 18:3 n-6 coincides with the fatty acid 20:0. The precise contribution of this saturated acid to the n-6 metabolite was later quantified by capilliary GLC to be 23.2±8.9 % (n=7). This facility was not available for all samples but on the small number that were analysed there was no systematic effect of cholesterol on the percent contribution of 20:0. This is note worthy as the overall trend is for the dietary cholesterol to lower the long chain SFA (16:0 and 18:0) in phospholipids. However, the factors that control phospholipid and fatty acid composition are far from being understood. It is therefore unwise to interpret indices of desaturation without actual measurements of the metabolic activity of a pathway. However, our results suggest that the index may adequately depict changes in 18:2 n-6 metabolism induced by large amounts of dietary cholesterol under highly controlled dietary conditions in the rat. Whether similar effects could have been observed with smaller amounts of cholesterol remains to be seen. It is also not clear whether these results can be extrapolated to man. Angelico *et al.* (1983) demonstrated that dietary intervention in hyper-cholesterolaemic patients increased the plasma 18:2 n-6/18:1 n-9 ratio and was associated with a 10 % decrease in plasma cholesterol. Unfortunately information on other n-6 fatty acids was not reported.

Fatty acids derived from 18:0 provide useful additional information. Decreased amounts of 18:0 and elevated amounts of 18:1 n-9 in microsomal phospholipids may indicate an elevated Δ9-desaturase activity. Further desaturation and elongation of 18:1 n-9 yields 20:3 n-9 (Mead acid) which is used as an indicator of EFA deficiency (Fulco and Mead, 1959). Mead acid, absent from the diet, is elevated in the cholesterol fed rats and may relate to increased 18:0 metabolism at the level of Δ9-, Δ6- and Δ5-desaturases. This may therefore support the finding of increased Δ6-desaturase activity. However, the metabolism of 18:1 n-9 by Δ6-desaturase was not examined formally.

Activity of Δ6-desaturase depends both on the amount of desaturase protein within the membrane as well as its surrounding membrane fluidity. The protocol used in this study was aimed at measuring maximal activity and thus the total amount of active desaturase protein. Garda and Brenner (1985) have shown dramatic changes in Δ6-desaturase activity as a result of enrichment of cholesterol into microsomal membranes *in vitro*. Thus demonstrating that activity of a distinct amount of desaturase protein can be altered by direct changes in membrane fluidity. However, changes at the level of transcription and translation of the desaturase protein could also occur under the influence of changes in insulin and related hormones.

A metabolic response to the cholesterol feeding may be to increase the actual amount of Δ6-desaturase protein in order to prevent the potential lack of throughput of 18:2 n-6. The level of Δ5-desaturase, however, does not seem to respond in a similar way by reference to the fatty acid ratios and may therefore be a time related event (i.e. the transcription and translation of this desaturase may not be triggered until a certain level of the precursor, 20:3 n-6, has accumulated). Nevertheless, actual measurements of Δ5-desaturase were not carried out.

It is important to remember that this study only investigated two tissues. As the liver is the major metabolic site for the formation and distribution of EFA desaturation and elongation products it is usually studied at the expense of other tissues. The scenario of depressed levels of long chain n-6 fatty acids may have occurred through depletion by excess output to extra-hepatic tissues via the plasma compartment. This may be the result of excessive influx into the liver of cholesterol that needs to be esterified. This area was not within the remit of the present investigation but it is apparent that it requires investigation.

Elevated liver to body weight ratio was one of the preliminary findings of this study. This has been shown by some but not all workers using similar cholesterol feeding protocols. Differences may depend upon the amount of cholesterol given as well as the amount and composition of the fat supplied. A dose response effect of

supplemental cholesterol has been documented (Tsai *et al.*, 1975), though some dietary experiments feeding a highly polyunsaturated fat along with 1 % cholesterol have found no such increase in liver weight (Muriana *et al.*, 1992). Garg *et al.* (1988a) fed diets of similar cholesterol and fatty acid composition to those described here for a duration of 28 days to male Sprague Dawley rats but contrary to the present findings no such effect on liver to body weight ratio was manifest. The control diet in the present study contained 0.02 % w/w cholesterol compared to the one of Garg *et al.* (1988a) that contained 0.12 %. It is feasible that the larger difference between control and cholesterol enriched diets in this study, increased the effectiveness of cholesterol at elevating the liver weight ratio. Indeed, studies with only low amounts of cholesterol (0.25 % w/w, Tsai *et al.*, 1975) have found increases in liver weight.

Thus in summary, dietary cholesterol in large amounts increased the maximum activity of Δ6-desaturase *in vitro*, but tissue fatty acid compositions suggest that the major effect was a reduction in the overall metabolism of 18:2 n-6 to 20:4 n-6 at the level of the Δ5-desaturase. If these results could be confirmed by direct measurements, then Δ6-desaturase activity should no longer be considered to be the major rate limiting step of 18:2 n-6 metabolism.

The effect of isolation stress, which may have additional modulatory effects within this model needs further examination. Measurement of phospholipid composition and of Δ6-desaturase activity at sub-optimal conditions may also shed more light on the effects of dietary cholesterol. These above proposed questions will be examined in Chapter 5.

Chapter 5

Linoleic acid metabolism in the rat
Effect of dietary cholesterol and isolation stress

5.1 Introduction

Dietary supplementation with cholesterol induced an increase not a decrease in liver microsomal Δ6-desaturase activity (Chapter 4). This observation was contrary to the results of other cholesterol feeding studies in the literature (Leikin and Brenner, 1987; Garg *et al.*, 1988a; Muriana *et al.*, 1992) but was in agreement with an index of Δ6-desaturase activity (i.e. 18:3 n-6/18:2 n-6 ratio in microsomal phospholipids) in these animals (Chapter 4).

A number of reasons might explain this contradictory finding. In some cholesterol supplementation studies, Δ6-desaturase activity is estimated using a large amount of microsomal protein with a small amount of substrate (Garg *et al.*, 1988a), a situation that could be sub-optimal. Furthermore, it is possible that non-esterified 18:2 n-6 in microsomal preparations may have diluted the specific activity of [1-^{14}C]18:2 n-6 in the assay *in vitro*. Levels of non-esterified fatty acids are rarely reported but large amounts could conceivably affect the Δ6-desaturase estimation leading to an erroneous result. Finally, animal housing conditions were regarded as an important factor in determining the activity of Δ6-desaturase in rat liver microsomes.

Therefore, some of the potential causes for the apparent controversy regarding the effect of dietary cholesterol supplementation are examined in this chapter. Microsomal phospholipid composition is also investigated to give more information on the changes that occur in the microsomal membrane as a result of cholesterol supplementation.

5.2 Methods

Male Sprague Dawley rats (n=24, ~115 g) were randomised into four groups. Half were fed the semi-synthetic diet (Section 2.2) while the other half were fed the same diet enriched with 2 % (w/w) cholesterol. These groups were sub-divided into two further groups of animals that were either group-housed (n=3 per cage) or housed individually (with no visual or social contact). Control and experimental diets were provided *ad libitum* for 27 days. Animals were examined regularly and weighed daily to ensure they were in good health for the duration of the experiment.

Rats were sacrificed under standard conditions (Section 2.3.1) and in the fed state to control for diurnal variations and to maximise Δ6-desaturase activity. Tissues were collected over two separate days to facilitate rapid sample processing. Microsomes were prepared as described in Section 2.3.1 and Δ6-desaturase activity was measured under the optimal conditions described in Section 2.4.8. In addition, Δ6-desaturase activity was measured under sub-optimal conditions with 5.0 mg microsomal protein and 200 nmol 18:2 n-6 (essentially as described by Garg *et al.*, 1988a).

Microsomal total and fractionated phospholipids were analysed as described in Sections 2.5.2 and 2.5.3, respectively. Microsomal total cholesterol, plasma lipids and plasma lipid fatty acid composition were determined as documented in Sections 2.5.7, 2.4.1 and 2.5.4 respectively. Adipose tissue triacylglycerol fatty acid composition was analysed as described in Section 2.5.6.

5.3 Results

5.3.1 General animal characteristics

Most of the animals remained healthy. The effect of isolation was evident as two animals (one from each of the two isolation groups) developed alopecia with some weight loss. The animal in the cholesterol fed group recovered quickly (after brief skin treatment with Dermisol cream, Smith Kline Beecham, Tadworth, Surrey, U.K.) but unfortunately the animal in the control (isolation) group had to be humanely destroyed. The results presented are therefore from a total of 23 rats. The growth rates of the four groups were in close agreement (Fig. 5.1) with no statistically significant differences manifest.

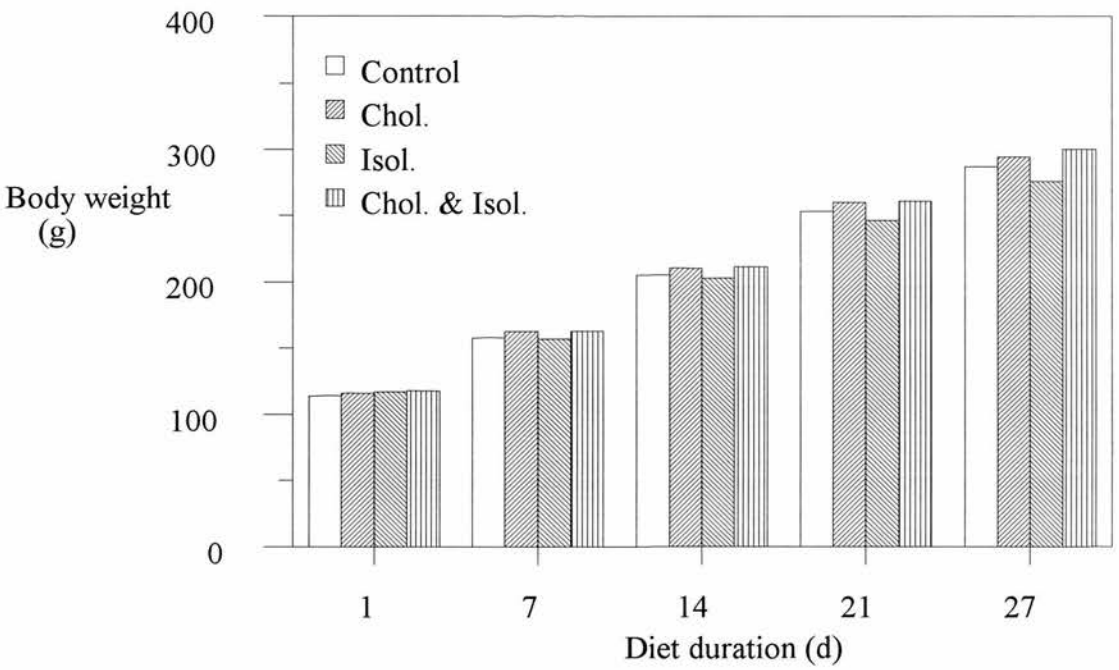


Figure 5.1 *Mean body weights during the experiment of rats fed a control or cholesterol enriched diet, with or without social isolation (No significant differences were observed)*

None of the four regimens had a significant effect on body weight, heart weight or heart to body weight ratio (Table 5.1). Yet, both liver weight and the ratio of liver to body weight were significantly elevated by cholesterol supplementation. Social

isolation in combination with cholesterol supplementation caused a significant increase in liver weight ($p<0.05$). Isolation itself had no significant effect on these variables.

Table 5.1 *Body weights, liver weight and heart weight of rats fed a control or cholesterol enriched diet, with or without social isolation*

Measurement	Control n=6	Chol. n=6	Isol. n=5	Chol. & Isol. n=6	ANOVA significance
Weight (g)					
Body					
Initial	114±6	116±7	117±3	117±4	n.s.
Final	290±15	296±27	278±10	303±17	n.s.
Difference	176±13	180±22	161±7	186±12	n.s.
Liver	12.0±0.8	14.4±2.0	11.7±0.7	15.6±1.2	C*** C/I*
Heart	1.08±0.10	1.11±0.12	1.07±0.07	1.11±0.06	n.s.
Relative weight (%)					
Liver/body	4.1±0.2	4.8±0.3	4.2±0.2	5.2±0.3	C***
Heart/body	0.37±0.02	0.38±0.01	0.39±0.02	0.37±0.01	n.s.

Values expressed as Mean±SD. ANOVA two-way analysis of variance. C cholesterol effect, C/I cholesterol and isolation interaction. * $p<0.05$, *** $p<0.001$.

5.3.2 Microsomal total phospholipid and cholesterol contents

Cholesterol feeding increased the total cholesterol content of liver microsomes in the two cholesterol supplemented groups as predicted (Table 5.2). Neither cholesterol feeding nor isolation had any effect on the total phospholipid content. The resultant cholesterol to phospholipid molar ratio was significantly increased by cholesterol supplementation. Isolation had no effect on these variables (Table 5.2).

Table 5.2 *Microsomal cholesterol, phospholipid and non-esterified fatty acid contents and Δ6-desaturase activities of rats fed a control or cholesterol enriched diet, with or without social isolation*

Measurement	Control n=6	Chol. n=6	Isol. n=5	Chol. & Isol. n=6	ANOVA significance
Cholesterol ξ	61±8	83±9	59±14	83±8	C***
Phospholipid ξ	408±33	424±33	400±16	401±40	n.s.
Cholesterol/phospholipid ψ	0.15±0.03	0.20±0.03	0.15±0.04	0.21±0.02	C**
Δ6-desaturase activity ζ	411±27	447±56	353±38	411±28	C** I**
Δ6-desaturase activity #	12±3	16±4	10±3	14±4	C**
Non-esterified 18:2 n-6 ξ	2.8±0.4	2.6±0.2	2.8±0.5	2.9±0.4	n.s.
Non-esterified 20:4 n-6 ξ	2.6±0.3	2.4±0.3	2.7±0.6	2.5±0.3	n.s.
Non-esterified 18:1 ξ	7.1±1.3	8.8±1.5	6.7±1.5	9.1±1.6	C**

Values expressed as Mean±SD. ANOVA two-way analysis of variance. C cholesterol effect, I isolation effect. ** $p<0.01$, *** $p<0.001$. ξ nmol/mg protein, ψ mol/mol, ζ Δ6-desaturase activity estimated with 0.5 mg microsomal protein (activity expressed as pmol/min/mg). # Δ6-desaturase activity estimated with 5.0 mg microsomal protein (activity expressed as pmol/min/mg).

5.3.3 Microsomal Δ6-desaturase activity

As expected, Δ6-desaturase activity assessed using our standard amount of 0.5 mg microsomal protein was significantly elevated by cholesterol supplementation (Table 5.2). This effect was apparent both with and without isolation. Furthermore, the cholesterol effect on Δ6-desaturase activity was still evident when assayed with 5.0 mg microsomal protein even though the activities were approximately 30-fold less than those exhibited with the optimised Δ6-desaturase assay system (Table 5.2). Isolation was effective at decreasing Δ6-desaturase activity when assessed with 0.5 mg microsomal protein either in the presence or absence of supplementary cholesterol. This effect, however, did not reach statistical significance when Δ6-desaturase activity was estimated with 5.0 mg microsomal protein, although a similar pattern was manifest. No cholesterol-isolation interactive effect was apparent with either of the two amounts of microsomal protein assessed.

Combining all individual results, there was a significant correlation for Δ6-desaturase activity estimated at the two concentrations of microsomal protein (r=0.673, $p<0.001$, Fig. 5.2).

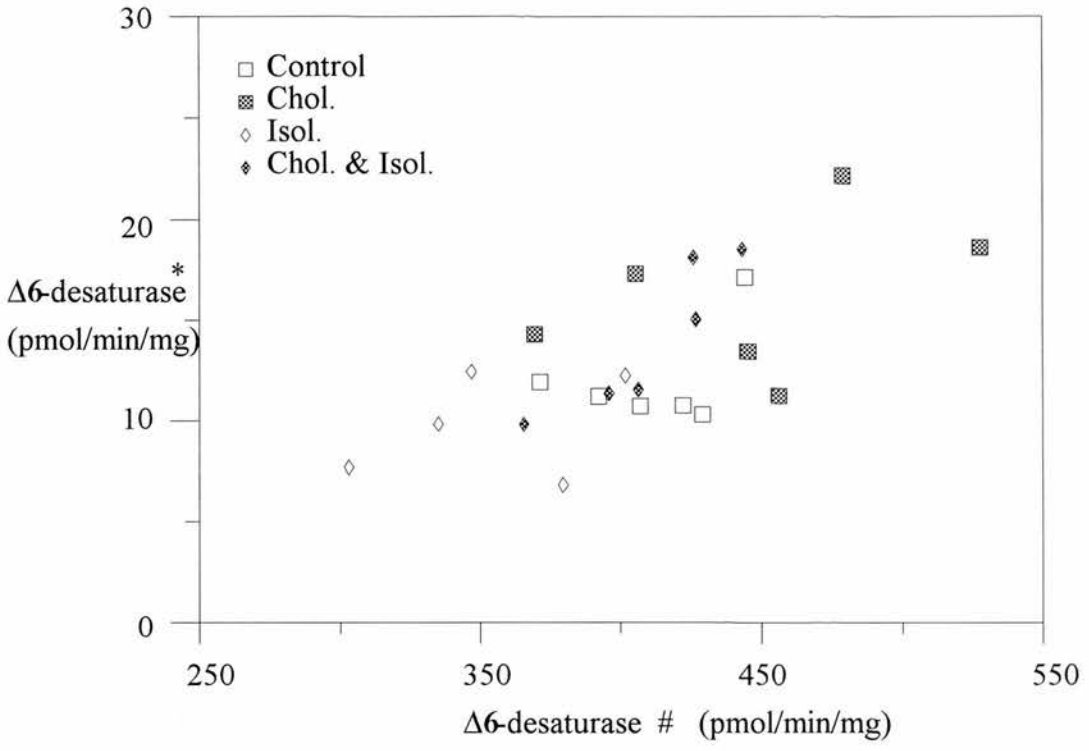


Figure 5.2 *Comparison of Δ6-desaturase activity estimated with 0.5 and 5.0 mg of microsomal protein of rats fed a control or cholesterol enriched diet, with or without social isolation. # Measured with 0.5 mg protein, * measured with 5.0 mg microsomal protein.*

Measurements of the non-esterified fatty acids, 18:2 n-6, 20:4 n-6 and 18:1 are shown in Table 5.2. The mean amount of non-esterified 18:2 n-6 in the microsomal fraction was approximately 3 nmol/mg microsomal protein and did not differ significantly between the four study groups. A similar result was evident for levels of non-esterified 20:4 n-6. In contrast, levels of non-esterified 18:1, a competitive inhibitor of 18:2 n-6 Δ6-desaturation, were significantly increased in microsomes obtained from the two cholesterol supplemented groups. Isolation had no effect on non-esterified fatty acid concentrations.

5.3.4 Microsomal total phospholipid fatty acid composition

As documented in the previous study (Chapter 4), widespread modifications to total phospholipid fatty acid composition resulted from cholesterol supplementation (Table 5.3). The results presented here are expressed as percentages to facilitate comparison with the earlier results. Microsomal phospholipid pool sizes did not differ in this study and so the use of percentages is valid.

Table 5.3 *Liver microsomal total phospholipid fatty acid composition of rats fed a control or cholesterol enriched diet, with or without social isolation*

Fatty acid species	Control n=6	Chol. n=6	Isol. n=5	Chol. & Isol. n=6	ANOVA significance
SFA					
14:0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	C**
16:0	14.2±0.9	13.1±0.7	13.6±0.3	13.1±0.5	C*
18:0	30.0±1.0	28.4±0.9	30.8±0.5	27.4±1.1	C** C/I*
MUFA					
16:1	0.5±0.1	0.8±0.1	0.5±0.1	1.0±0.2	C** C/I*
18:1	7.5±0.6	11.4±0.9	7.6±0.3	12.0±1.1	C***
20:1	0.1±0.0	0.3±0.0	0.1±0.0	0.3±0.1	C***
PUFA (n-6)					
18:2	10.2±0.7	11.8±0.9	10.0±0.5	12.3±0.6	C***
20:2	0.1±0.0	0.2±0.0	0.1±0.0	0.2±0.0	C***
18:3 #	0.2±0.2	0.1±0.0	0.1±0.0	0.1±0.0	n.s.
20:3	1.7±0.1	2.3±0.1	1.6±0.2	2.5±0.2	C***
20:4	26.6±0.8	24.1±1.1	26.7±0.6	23.6±1.1	C***
22:4	0.4±0.1	0.3±0.0	0.4±0.0	0.3±0.0	C***
22:5	1.6±0.6	0.9±0.3	1.6±0.3	0.9±0.2	C***
PUFA (n-3)					
20:5	0.1±0.0	0.2±0.0	0.1±0.1	0.2±0.0	C***
22:5	0.6±0.1	0.5±0.1	0.5±0.1	0.5±0.0	n.s.
22:6	5.7±0.5	4.6±0.2	5.7±0.3	4.7±0.3	C***
PUFA (n-9)					
20:3	0.5±0.1	0.8±0.1	0.5±0.2	0.9±0.2	C***
Σ SFA	44.3±0.5	41.6±0.9	44.4±0.3	40.7±0.9	C***
Σ MUFA	8.2±0.6	12.5±1.0	8.3±0.4	13.3±1.3	C***
Σ n-6	40.7±0.7	39.6±0.6	40.4±0.2	39.7±0.5	C**
Σ n-3	6.4±0.4	5.3±0.1	6.3±0.2	5.3±0.2	C***
DBI	190±5	180±3	189±2	180±3	C***

Values expressed as % (w/w) of total fatty acids (Mean±SD). ANOVA two-way analysis of variance, C cholesterol effect, C/I cholesterol and isolation interactive effect. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. # Unresolved peak contained unspecified amounts of 20:0.

There was a significant increase in the MUFA fraction at the expense of total levels of SFA. Total amounts of n-6 and n-3 fatty acids were also significantly reduced by

cholesterol supplementation. N-6 and n-3 fatty acid class compositional changes were generally similar to those demonstrated in Chapter 4. An exception to this was 18:3 n-6 which was not altered. This was due to a high level of 18:3 n-6 in one of the control samples analysed by packed column GLC. Subsequent analysis by capillary column GLC confirmed that the peak was 94 % (w/w) 18:3 n-6. The result was therefore included in the statistical analysis.

5.3.5 Microsomal phospholipid composition

Phosphatidylcholine (PC) constituted the principal microsomal phospholipid sub-class amounting to over 60 % of the total phospholipid present in each of the four experimental groups (Table 5.4). Cholesterol supplementation but not isolation increased the PC component when expressed in absolute amounts (nmol/mg microsomal protein). When presented as a percentage of total phospholipids, however, isolation caused a statistically significant reduction in the PC contribution.

Table 5.4 *Proportions of PC, PE and PI in liver microsomal phospholipids of rats fed a control or cholesterol enriched diet, with or without social isolation*

Phospholipid type	Control n=6	Chol. n=6	Isol. n=5	Chol. & Isol. n=6	ANOVA significance
PC ξ	266±25	305±30	255±15	274±30	C*
PC ζ	65.1±3.5	71.7±1.6	63.8±1.8	68.2±1.9	C*** I*
PE ξ	68±17	63±8	60±11	54±8	n.s.
PE ζ	16.5±2.7	14.9±1.9	14.9±2.4	13.6±1.8	n.s.
PI ξ	18±3	17±3	17±2	18±2	n.s.
PI ζ	4.3±0.3	4.1±0.7	4.2±0.4	4.5±0.2	n.s.
PC/PE (mol/mol)	4.0±0.7	4.9±0.7	4.4±0.8	5.1±0.8	C*

Values expressed as Mean±SD. ANOVA two-way analysis of variance. C cholesterol effect, I isolation effect, * $p<0.05$, *** $p<0.001$. ξ nmol/mg microsomal protein, ζ mol %.

No changes in either phosphatidyl ethanolamine (PE) or phosphatidyl inositol (PI) were apparent when expressed as either absolute amounts or as percentages. The PC/PE ratio, which is frequently used in cholesterol feeding studies as an indicator of

phospholipid metabolism, was also significantly increased by cholesterol supplementation while isolation had no effect on this parameter.

5.3.6 Microsomal phospholipid sub-class fatty acid composition

Analysis of n-6 fatty acid composition of three sub-classes of microsomal phospholipids revealed, as predicted, that many of the alterations were again cholesterol induced (Table 5.5).

Table 5.5 *Liver microsomal phospholipid sub-class n-6 fatty acid composition from rats fed a control or cholesterol enriched diet, with or without social isolation*

Phospholipid fraction and fatty acid species	Control n=6	Chol. n=6	Isol. n=5	Chol. & Isol. n=6	ANOVA significance
PC					
18:2	12.6±1.4	14.6±1.2	12.7±0.7	15.2±1.0	C***
20:2	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	C*** I*
18:3 #	0.1±0.0	0.2±0.0	0.1±0.0	0.2±0.0	C***
20:3	1.9±0.2	2.6±0.2	1.9±0.2	2.7±0.2	C***
20:4	22.9±1.9	21.2±1.5	22.5±0.8	19.4±1.4	C**
22:4	0.3±0.0	0.2±0.0	0.2±0.0	0.2±0.0	C** C/I*
22:5	1.4±0.5	0.8±0.3	1.4±0.3	0.8±0.2	C**
Σ n-6	39.4±1.1	39.7±0.7	39.0±0.2	38.8±0.6	I*
DBI	173±9	171±5	171±3	164±5	n.s.
PE					
18:2	6.2±0.7	7.8±0.5	6.4±0.8	8.0±0.3	C***
20:2	0.0±0.0	0.1±0.0	0.0±0.0	0.1±0.0	C***
18:3 #	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1	n.s.
20:3	0.9±0.1	1.3±0.1	0.8±0.2	1.3±0.1	C***
20:4	28.4±0.7	28.6±0.6	28.1±0.9	27.2±0.5	I**
22:4	1.2±0.4	0.9±0.2	1.1±0.2	1.3±0.4	n.s.
22:5	2.6±1.0	1.6±0.5	2.6±0.6	1.5±0.3	C**
Σ n-6	39.5±0.5	40.5±0.8	39.2±0.4	39.6±0.6	C* I*
DBI	220±6	220±3	217±4	212±2	I**
PI					
18:2	2.3±0.5	2.5±0.3	1.8±0.2	2.0±0.4	I**
18:3 #	0.2±0.2	0.1±0.1	0.2±0.1	0.1±0.1	n.s.
20:3	1.3±0.1	2.5±0.4	1.4±0.2	2.6±0.4	C***
20:4	38.9±2.0	36.2±0.6	39.6±0.9	36.0±1.6	C***
22:4	0.5±0.1	0.5±0.0	0.4±0.1	0.6±0.1	C**
22:5	0.4±0.1	0.6±0.2	0.4±0.1	0.6±0.2	C**
Σ n-6	43.6±1.6	42.5±0.7	43.7±0.6	41.8±1.1	C**
DBI	186±6	188±2	186±3	185±4	n.s.

Values expressed as % (w/w) of total fatty acids (Mean±SD). ANOVA two-way analysis of variance, C cholesterol effect, I isolation effect, C/I cholesterol and isolation interactive effect. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. PI fraction contained no detectable 20:2 n-6. # Unresolved peak contained unspecified amounts of 20:0.

For PC, the pattern of increased 18:2, 18:3 and 20:3 with decreased levels of 20:4, 22:4 and 22:5 was similar to that observed in total phospholipids. Cholesterol also increased 20:2 in normally housed rats (0.15±0.03 and 0.19±0.03 for control and cholesterol supplemented, respectively) and in isolated rats (0.16±0.02 and 0.24±0.03 control diet and cholesterol supplemented, respectively). Isolation by itself also increased 20:2 ($p=0.02$). The total amount of n-6 fatty acids present was also significantly lowered by isolation while cholesterol supplementation had no effect on this measurement. Despite all these fatty acid alterations the DBI remained unchanged.

Microsomal PE n-6 fatty acid composition was significantly modified both by cholesterol and isolation. Cholesterol increased levels of 18:2, 20:2 and 20:3 while only 22:5 was lowered. A notable exception to these cholesterol-induced modifications was 20:4 that remained unchanged by the cholesterol supplementation but was significantly lowered by isolation. Of interest, 18:3 and 22:4 were not modified by either cholesterol supplementation or isolation.

Microsomal PI n-6 fatty acid composition was similarly affected by the cholesterol supplementation. Cholesterol-induced increases were discernible for 20:3, 22:4 and 22:5 while 18:3 remained unchanged. Cholesterol induced decreases occurred for both 20:4 and total n-6 fatty acid content. No cholesterol supplementation effect was apparent on the small amount of 18:2 present but isolation had a marked and significant lowering effect on this fatty acid. The DBI remained unchanged.

5.3.7 Plasma lipid fatty acid compositions

As the total amount of each plasma lipid fraction differed between the four groups, presumably owing to different lipoprotein patterns, the results are expressed as total nmol of n-6 fatty acids present in each of the three fractions (Table 5.6).

Table 5.6 *Plasma phospholipid, cholesterol ester and triacylglycerol n-6 fatty acid composition of rats fed a control or cholesterol enriched diet, with or without social isolation*

Plasma lipid and fatty acid species	Control n=6	Chol. n=6	Isol. n=5	Chol. & Isol. n=6	ANOVA significance
Phospholipid ‡	1.35±0.29	1.62±0.29	1.38±0.14	1.84±0.30	C**
PUFA (n-6)					
18:2	731±159	895±149	741±71	1026±178	C**
20:3	48±12	69±12	46±8	89±13	C/I*
20:4	544±118	570±112	546±61	645±118	n.s.
22:4	9.5±2.3	6.1±3.2	7.7±0.9	7.7±0.9	n.s.
22:5	30±13	20±7	30±8	21±4	C*
Σ n-6	1362±276	1560±262	1371±135	1788±302	C*
Cholesterol ester ‡	1.68±0.30	2.19±0.83	1.80±0.23	2.72±0.40	C**
PUFA (n-6)					
18:2	408±81	374±116	430±57	478±78	n.s.
18:3	7.4±1.5	9.8±4.0	8.8±1.7	9.0±2.0	n.s.
20:3	12±3	12±4	15±4	17±4	I*
20:4	853±170	568±231	906±112	701±186	C**
22:4	2.3±2.0	2.8±1.9	2.4±1.6	3.5±1.8	n.s.
22:5	3.4±1.9	2.1±2.5	3.9±1.7	1.5±1.1	C*
Σ n-6	1286±237	969±345	1366±163	1210±262	C*
Triacylglycerol ‡	0.96±0.48	2.04±1.48	1.34±0.57	1.76±0.79	n.s.
PUFA (n-6)					
18:2	317±160	589±417	446±201	510±213	n.s.
20:3	2.4±1.4	9.6±8.9	3.2±2.1	6.2±2.1	C*
20:4	30±13	43±35	40±19	30±14	n.s.
22:4	6.8±2.7	12±10	7.8±3.2	8.8±3.0	n.s.
22:5	11±6	17±16	15±10	12±4	n.s.
Σ n-6	367±177	670±486	513±232	567±233	n.s.

All fatty acids expressed as μmol/l (Mean±SD). ‡ Expressed as mmol/l. ANOVA two-way analysis of variance, C cholesterol effect, I isolation effect, C/I cholesterol and isolation interactive effect. * $p<0.05$, ** $p<0.01$.

There was a significant increase in plasma total phospholipid content in cholesterol fed animals. Within this fraction, the amount of esterified 18:2 was elevated by cholesterol supplementation but no significant effect was evident for either 20:4 or 22:4. Lower amounts of 22:5 were present in cholesterol supplemented rats despite a cholesterol-induced increase in the total amount of n-6 fatty acids. Interestingly, cholesterol in combination with isolation increased the level of 20:3 n-6 (Table 5.6).

Cholesterol supplementation had no effect on the total amount of 18:2, 18:3 and 22:4, however, both 20:4 and 22:5 were significantly decreased despite increased cholesterol ester levels induced by cholesterol feeding. Isolation by itself increased

levels of 20:3 n-6 in both socially deprived groups but the total amount n-6 fatty acids present in the cholesterol esters was decreased significantly by the cholesterol supplement.

Triacylglycerol fatty acid modifications were limited and large variances observed for this data may have been responsible. However, omission of one sample with a particularly high triacylglycerol content (>4 mM) had no effect on the pattern of significant results. Nevertheless, 20:3 n-6 was elevated by cholesterol supplementation within this fraction.

5.3.8 Adipose tissue triacylglycerol fatty acid composition

Small changes occurred in the fatty acid composition of adipose tissue triacylglycerols as a result of the cholesterol supplementation. No such modifications occurred as a result of social isolation (Table 5.7).

Table 5.7 *Adipose tissue triacylglycerol fatty acid composition of rats fed a control or cholesterol enriched diet, with or without social isolation*

Fatty acid species	Control n=6	Chol. n=6	Isol. n=5	Chol. & Isol. n=6	ANOVA significance
SFA					
14:0	2.3±0.3	2.1±0.1	2.2±0.1	2.1±0.1	C*
16:0	22.9±0.7	21.3±0.7	23.3±1.2	21.6±0.5	C***
18:0	8.9±1.1	8.8±0.9	8.3±1.6	8.4±0.7	n.s.
MUFA					
16:1	6.0±1.0	5.6±0.5	6.5±1.4	5.8±0.3	n.s.
18:1	45.6±0.6	47.9±1.0	45.8±0.6	48.2±0.7	C***
20:1	1.2±0.0	1.3±0.1	1.2±0.1	1.3±0.1	C**
PUFA (n-6)					
18:2	11.0±0.5	10.8±0.3	10.7±0.9	10.5±0.3	n.s.
20:3	0.1±0.0	0.0±0.0	0.1±0.0	0.1±0.0	n.s.
20:4	0.1±0.0	0.1±0.0	0.1±0.1	0.1±0.0	n.s.
PUFA (n-3)					
18:3	0.3±0.0	0.3±0.0	0.3±0.1	0.3±0.1	n.s.
Σ SFA	34.8±1.1	32.8±1.3	34.4±0.5	32.6±0.8	C***
Σ MUFA	52.7±1.3	54.8±1.2	53.5±1.2	55.3±0.9	C**
Σ n-6	11.3±0.6	11.0±0.3	10.9±0.8	10.7±0.3	n.s.
Σ n-3	0.4±0.1	0.3±0.0	0.3±0.1	0.4±0.1	n.s.
DBI	77±1	78±1	77±1	79±1	C**

Values expressed as % (w/w) of total fatty acids (Mean±SD). ANOVA two-way analysis of variance, C cholesterol effect, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Both 14:0 and 16:0 were significantly lowered by the cholesterol supplement while both 18:1 and 20:1 were elevated culminating in distinct reductions and elevations in the SFA and MUFA components, respectively. The level of 20:3 n-6 was decreased slightly by the cholesterol regimen but this did not achieve statistical significance (p=0.072).

5.3.9 Microsomal phospholipid fatty acid ratios

Indices for the desaturases are shown in Table 5.8. Contrary to the previous cholesterol feeding experiment no significant effect of the feeding protocol was demonstrated for the 18:3 n-6/18:2 n-6 Δ6-desaturase index. In addition, isolation had no significant effect on this ratio.

Table 5.8 *Indices of desaturase activity derived from microsomal phospholipid fatty acid composition and triene/tetraene ratios of rats fed a control or cholesterol enriched diet, with or without social isolation*

Fatty acid ratio	Control n=6	Chol. n=6	Isol. n=5	Chol. & Isol. n=6	ANOVA significance
n-6					
18:3/18:2 (Δ6)	0.021±0.018	0.010±0.002	0.009±0.000	0.009±0.001	n.s.
20:4/20:3 (Δ5)	16.0±1.6	10.5±1.0	16.7±2.2	9.5±0.9	C***
22:5/22:4 (Δ6)	4.1±1.2	3.4±0.8	4.3±1.1	3.2±0.5	C*
18:2 D&E	3.01±0.32	2.37±0.28	3.05±0.19	2.25±0.19	C***
n-9					
18:1/18:0 (Δ9)	0.258±0.025	0.402±0.045	0.248±0.014	0.438±0.058	C***
20:3 n-9/20:4 n-6	0.018±0.003	0.032±0.005	0.018±0.006	0.036±0.008	C***

Values expressed as Mean±SD. * $p<0.05$, *** $p<0.001$ *vs.* control. Indices for Δ6- and Δ5-desaturases were calculated using the n-6 fatty acid series. 18:2 D (Desaturation) & E (Elongation) refers to (18:3 n-6 + 20:2 n-6 + 20:3 n-6 + 20:4 n-6 + 22:4 n-6 + 22:5 n-6)/18:2 n-6.

The effect of cholesterol feeding on the total metabolites derived from the desaturation and elongation of 18:2 n-6 was highly evident and related mainly to the depressive effect of cholesterol on the 20:4 n-6/20:3 n-6 Δ5-desaturase index. A small

but significantly decreased 22:5 n-6/22:4 n-6 Δ6-desaturase index was also evident. Once again cholesterol feeding increased the triene/tetraene ratio, while isolation had no effect on any of these variables.

5.4 Discussion

This study demonstrated once again that Δ6-desaturase activity was elevated by dietary cholesterol supplementation. Furthermore, the effect of cholesterol enrichment was still evident, though much less strongly, when Δ6-desaturase activity was assayed in sub-optimal conditions with 5.0 mg microsomal protein and 200 nmol substrate. These activities, however, were markedly lower (30-fold) and associated with a larger group variance.

It had been speculated that the isolation protocol used by Garg *et al.* (1988a), to assess individual dietary intakes, could have had an additional modulatory effect on the activity of Δ6-desaturase. Indeed, it was clear that isolation reduced Δ6-desaturase activity in rats fed either control or cholesterol supplemented diets when assayed under optimal conditions. Yet, with 5.0 mg microsomal protein the effect of isolation was no longer apparent and must relate to the larger error associated with this measurement. It was evident, however, that there was no interaction between the effects of cholesterol feeding and social isolation stress.

The depressive effect of stress hormones on rat liver microsomal Δ6-desaturase activity has been documented *in vivo* with the administration of pharmacological doses of adrenaline and hydrocortisone (De Gómez Dumm *et al.*, 1976; De Gómez Dumm *et al.*, 1979). Isolation of rats increases urinary catecholamine excretion (Crawford and Law, 1958), raises blood pressure (Huang *et al.*, 1990; Jiménez and Fuentes, 1993) and may elevate corticosterone levels (Apple *et al.*, 1993). The latter hormone reduces protein synthesis and may have affected the synthesis of Δ6-desaturase by this route (Marra *et al.*, 1986). Alternatively, raised circulating adrenaline levels may have decreased the activity of the enzyme mediated through cAMP (De Gómez Dumm *et*

al., 1976; Mills *et al.*, 1994). Interestingly, a recent investigation, unpublished at the time of our study, examining Δ6-desaturase activity in normotensive (Wistar Kyoto) and spontaneously hypertensive rats supports our observations of a psychosocially induced decrease in the activity of Δ6-desaturase (Mills *et al.*, 1994). The present study, however, goes further and demonstrates that microsomal total phospholipid fatty acid composition does not mirror the isolation induced reduction in Δ6-desaturase activity. Nevertheless, the level of 18:2 n-6 in the microsomal PI fraction and the amount of 20:4 n-6 in the PE fraction were reduced by isolation.

Within the assay system, dilution of the radioactive substrate by endogenous non-esterified fatty acids could affect the apparent activity of Δ6-desaturase. The results demonstrated that the level of non-esterified 18:2 n-6 was similar in all groups. Results are normally corrected for this lowering of [1-^{14}C]18:2 n-6 specific activity but there was no systematic bias introduced as a result. Nevertheless, the true activity of Δ6-desaturase may be some 1-2 % higher.

Microsomal non-esterified 18:1 levels were higher in cholesterol fed rats compared with control. Large amounts of 18:1 n-9 can act competitively with 18:2 n-6 for Δ6-desaturation. A 50 % reduction in Δ6-desaturase activity has been documented using a 2.5:1 molar ratio of 18:1 n-9 to 18:2 n-6 (Mahfouz *et al.*, 1980). Lower reductions in activity (~10 %) have been demonstrated with equimolar concentrations of inhibitor and substrate (Brenner and Peluffo, 1966). The inhibitor-substrate ratio in this study was approximately 1:20 and therefore the inhibitory effect would be negligible. Interestingly, Δ6-desaturase activity in microsomes from cholesterol fed rats was increased despite this 30 % elevation in non-esterified 18:1.

Similar problems associated with non-esterified fatty acids were also considered for 18:3 n-6 and 20:4 n-6. The end products of Δ6- and Δ5-desaturation play important roles as a feed back inhibitors of desaturation (Leikin and Brenner, 1986; Leikin and Brenner, 1989b). Levels of non-esterified 18:3 n-6 were below the level of detection and amounts of 20:4 n-6 were not significantly different between all groups. Thus the

effect of cholesterol supplementation on Δ6-desaturation cannot be necessarily explained in this way.

This experiment demonstrated that a large number of microsomal membrane modifications occurred as a result of dietary cholesterol supplementation. Microsomal total cholesterol, cholesterol/phospholipid ratio, PC content and PC/PE ratio were all increased in cholesterol fed animals. In contrast, alterations to total phospholipid fatty acid composition culminated in a decreased DBI. Within membranes cholesterol is orientated parallel with phospholipid acyl chains which increases membrane ordering and rigidity as demonstrated using steady state fluorescence anisotropy (Brenner, 1990; Muriana *et al.*, 1992) and pyrene excimer formation (Brenner, 1990). One of the observed responses to increased membrane cholesterol was an augmentation in membrane PC. This is in agreement with the findings of Leikin and Brenner (1987) and Muriana *et al.* (1992) although Garg *et al.* (1988a) showed no effect of dietary cholesterol enrichment on phospholipid composition. An increase in PC is associated with an elevated activity of CTP:phosphocholine cytidylyltransferase (Hirata and Axelrod, 1980; Lim *et al.*, 1983) and is thought to provide a more thermodynamically favourable membrane state. Indeed, intermolecular head group interaction is greater for bilayers containing PE compared to those containing PC due to a greater electrostatic attraction (Yeagle, 1985) and as a consequence incorporation of cholesterol into membranes containing PE is thermodynamically less favourable than those containing PC. Interestingly, increased levels of PE in mouse fibroblasts have a greater membrane viscosity compared to controls (Esko *et al.*, 1977) and dietary PE supplementation in rats decreases Δ6-desaturase activity and is associated with an elevated membrane PE content (Imaizumi *et al.*, 1989). Infact, the increase in PC may be viewed as a homeostatic mechanism to counteract the cholesterol-induced bulk rigidity of membranes.

Changes in PC/PE ratio induced by membrane sterol incorporation have been studied in the context of altered desaturase activities. Cholesterol incorporation *in*

vitro increases the activity of Δ5-, Δ6- and Δ9-desaturases by a viscotropic effect where there is no change in the PC/PE ratio (Garda and Brenner, 1985; Brenner, 1990). Cholesterol enrichment *in vivo* generally causes an increase in PC/PE ratio which is associated with a decrease in Δ5- and Δ6-desaturases and an increase in Δ9-desaturase (Leikin and Brenner, 1987). In effect, the increase in the PC/PE ratio attempts to fluidize the membrane as a compensatory mechanism (Brenner, 1990). Yet, when rats are fed 5% (w/w) phytosterol (3 % β-sitosterol and 2 % campesterol) no change in PC/PE ratio occurs and the activity of all the desaturases is increased (Leikin and Brenner, 1989a). It is evident therefore that the increase in the PC content may prevent the viscotropic regulation.

The role of cholesterol has also been examined by investigating dietary cholesterol removal (Leikin and Brenner, 1988). Their study showed that even after one day microsomal membrane modifications begin to occur. Of interest, no significant changes were observed for the molar PC/PE or cholesterol/phospholipid ratios or Δ5- and Δ6-desaturase activities. Nevertheless, the n-6 fatty acid composition of microsomal total phospholipids was modified indicating that EFA metabolism had been up-regulated. It is therefore pertinent that other factors must be considered in the regulation of phospholipid fatty acid composition which do not necessarily relate to the activity of the desaturases.

Although cholesterol fed animals had a greater Δ6-desaturase activity this was not reflected in the microsomal phospholipid index of 18:2 n-6 desaturation and elongation. It was considered that this may partially be due to a greater export of long chain n-6 fatty acids, especially 20:4 n-6, into the plasma compartment. Plasma cholesterol ester content was increased in both groups fed cholesterol but this was not paralleled by an increased proportion of n-6 fatty acids esterified to cholesterol. Indeed, the level of 20:4 n-6 esterified to cholesterol was reduced in cholesterol fed rats but no differences were observed in the amount of 20:4 n-6 in phospholipids Microsomal phospholipid composition cannot therefore be explained by an increased

export of 18:2 n-6 desaturation and elongation products to the plasma pool. Alternatively, it is possible that cholesterol ester accretion in the liver took place to remove large pools of free cholesterol. In this case a large influx of peroxidisable material may have overwhelmed the tissue antioxidant protection mechanisms (Tsai, 1975). This in turn may have increased the oxidation of fatty acids such as 20:4 n-6 and therefore explain the lower levels. However, this aspect of the work was not examined formally.

In summary, isolation stress was not the explanation for the decrease in microsomal Δ6-desaturase activity in Garg's earlier cholesterol supplementation study and the increase in our study. Indeed, isolation did not interact with the effect of cholesterol supplementation on n-6 fatty acid composition or microsomal cholesterol content in this study.

This work therefore still raises important questions to be answered. It is apparent that the assay system *in vitro* may not reflect the situation *in vivo*. Factors such as an overwhelming amount of substrate and cofactors with the absence of cellular controlling factors, including cytosolic fatty acid binding proteins, may explain these discrepancies. It is this aspect of 18:2 n-6 metabolism *in vivo*, conducted by Zevenbergen and Houtsmuller (1989), that has brought a new impetus to the study of EFA metabolism. This kind of work has not been examined in the light of cholesterol feeding and will be the subject for examination in Chapter 6.

Chapter 6

Linoleic acid metabolism in the cholesterol fed rat in vivo

6.1 Introduction

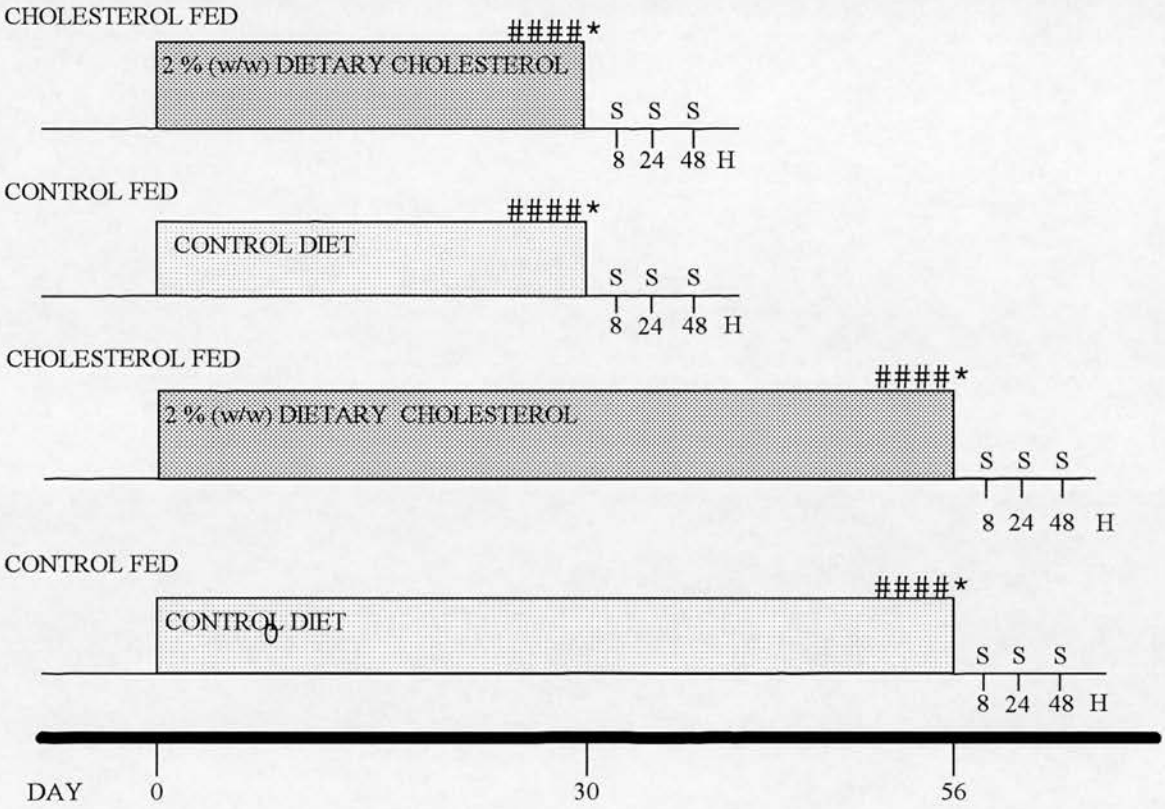
Dietary cholesterol enrichment increased rat liver microsomal Δ6-desaturase activity in Chapter 4. This finding was reproduced in Chapter 5 at both high and low amounts of microsomal protein (5.0 and 0.5 mg, respectively). Although this result was in agreement with the Δ6-desaturase fatty acid ratio (18:3 n-6/18:2 n-6) in Chapter 4, the result was contrary to other workers using a similar protocol of cholesterol enrichment *in vivo* (Garg *et al.*, 1988a). This led onto the belief that the findings could be due to an *in vitro* effect. It was considered that the results obtained could have been due to a very unphysiological situation in which overwhelming amounts of substrate and cofactors can be used solely for desaturation purposes. A situation that is not representative of that *in vivo* where the amount of substrate is low and many other metabolic fates for the substrate are available, such as fatty acid β-oxidation and esterification into complex membrane lipids.

This study was therefore undertaken to assess the effects of dietary cholesterol on 18:2 n-6 metabolism *in vivo*, in particular, in liver microsomes. The assessment of microsomal Δ6-desaturase activity *in vitro*, as employed in the previous cholesterol feeding experiments, was used as a comparison. In addition, to examine whether any particular effect was specific for Δ6-desaturase, the role of dietary cholesterol supplementation on 18:0 metabolism by Δ9-desaturase was also assessed *in vivo* and *in vitro*. This additional part of the study was carried out simultaneously within the same control and cholesterol fed rats.

6.2 Methods

Four groups of male, Sprague Dawley rats (n=18 per group, ~80 g) were purchased in order to phase this large feeding and oral dosing experiment. All rats were group-housed (n=3) and fed their appropriate diets *ad libitum*. The study design is shown in Figure 6.1.

Figure 6.1 *Design of experiment* ξ



ξ Duration of feeding either 30 or 56 d, # sham oral dosing, * dosing with [1-^{14}C]18:2 n-6 and [11,12-^{3}H]18:0, S blood samples, sacrifice and tissue excision (either 8, 24 or 48 h after the radioactive oral dose, n=6 at each time point). Design details not to scale.

To limit systematic variations in the procedures used in this large study, control and experimental rats were fed, sacrificed and analysed at the same time. Thus, each group of eighteen rats was randomly divided into two groups of nine and one half fed the control diet and the other half the same diet enriched with 2 % (w/w) cholesterol (see Section 2.2). They were fed for either 30 or 56 d. During the last week of the

feeding protocol rats were given sham oral dosing on four consecutive days to familiarise them with the procedure. No actual dose of oil was given at these times, although the oral dosing tube was lubricated with olive oil to facilitate the process.

The day before oral dosing with [1-^{14}C]18:2 n-6 and [11,12-^{3}H]18:0, groups were fasted for 21 h to empty the animals' stomachs (to facilitate uptake of fatty acids). The next day rats were transferred to an area designated for radioactivity (within the animal house) and dosed transoesophageally at 1230 hours with 30 μCi [11,12-^{3}H]18:0 (prepared as described in Section 2.6) and 20 μCi [1-^{14}C]18:2 n-6 (CFA 104, Amersham International, Amersham, U.K.). The radioactive fatty acids were dissolved in 0.2 ml olive oil (Lipid Teknik, Stockholm, Sweden) and administered to rats using an infant feeding tube (8G, 100 mm, Portex Ltd., Hythe, Kent, U.K.) attached to a 1.0 ml calibrated syringe (Becton Dickinson, Dublin, Ireland). The coefficient of variation of oil administration was determined by weighing the dose delivered into a test-tube was 3.1 % (n=10). All rats were then provided with their respective diets and water *ad libitum*.

Eight h after oral dosing with radioactivity, the first group (three rats from each of the two dietary regimens) were anaesthetised (60 mg/kg body weight, Sagatal®, Rhône Mérieux Ltd., Harlow, Essex, U.K.). Blood was withdrawn from the heart using a heparinised 10 ml syringe (Becton Dickinson, Dublin, Ireland) and plasma prepared and stored as described in Section 2.3.2. Rats were sacrificed by heart excision (under anaesthesia) and both liver and adipose tissue were removed and processed as described in Sections 2.3.1 and 2.3.2, respectively. Exactly the same procedures were employed for animals at 24 and 48 h after the radioactive oral dose.

Freshly prepared plasma aliquots (40 μl) were counted (20 min) in duplicate with 10 ml scintillant as described in Section 2.7.1. Plasma lipid classes (phospholipid, cholesterol ester, triacylglycerol and non-esterified fatty acid) were extracted and assessed for ^{3}H and ^{14}C (see Section 2.7.1). Plasma lipids (triacylglycerol and total cholesterol) were measured as described (Section 2.4.1). Plasma lipid fatty acid

composition was measured as outlined in Section 2.5.4. Microsomes were prepared (see Section 2.3.1) and Δ6-desaturase activity was measured under the optimal conditions described in Section 2.4.8. Owing to the nature of the study, all microsomal preparations contained different amounts of [^{14}C]triene and [^{14}C]tetraene fatty acids that might interfere with accurate Δ6-desaturase activity measurement *in vitro*. Therefore, for each sample, microsomes (0.5 mg protein) were added to incubation medium pre-treated with KOH-methanol and taken through the Δ6-desaturase estimation. The blank value derived from this (i.e. counts in the triene and tetraene bands adjusted for recovery) was subtracted from the actual Δ6-desaturase measurement for each sample. The mean blank value of the 72 duplicate Δ6-desaturase analyses carried out in this study was equivalent to a Δ6-desaturase activity of 106±16 pmol/min/mg microsomal protein (range 74-144).

Microsomal phospholipids were extracted, transmethylated and analysed for fatty acid composition and radioactivity as described in Sections 2.5.2 and 2.7.2. Microsomal total cholesterol was assessed as in Section 2.5.7. Adipose triacylglycerol fatty acid composition and radioactivity were analysed as described (Sections 2.5.6 and 2.7.3).

6.3 Results

The main purpose of this experiment was to elucidate the effect of cholesterol supplementation on [1-^{14}C]18:2 n-6 and [11,12-^{3}H]18:0 metabolism *in vivo*. The effect of extending the cholesterol feeding protocol to 56 d was included as no studies have examined the effect of this level of dietary cholesterol enrichment beyond 28 d. The effects of dietary cholesterol supplementation for 56 d were generally similar to the effects observed with the 30 d supplementation. Therefore to simplify result description, particularly regarding the fatty acid data, only information from rats fed control and cholesterol enriched diets for 56 d is described. Data for rats fed the respective diets for 30 d are presented in appendix E.

6.3.1 General background results

The data described in this section generally support the findings of the previous two cholesterol feeding studies. An abbreviated form of these non-radioactive results is presented to highlight some of the important changes that occurred.

6.3.1.1 Animal characteristics

All animals remained healthy for the duration of the experiment. Growth rates were comparable between control and cholesterol supplemented groups with no significant differences evident (data not shown). Fasting for 21 h reduced rat body weight by approximately 8 %. Body weights steadily increased on refeeding following oral administration with radioactive fatty acids (Table 6.1).

Table 6.1 *Body and liver weights of rats fed a control or cholesterol enriched diet for 56 days at 8, 24 and 48 h after administration of radioactive fatty acids*

Measurement	Time (h)	Control	Chol.	Significance
Weight (g)				
Body	8	311±28	332±46	n.s.
	24	324±31	321±43	n.s.
	48	333±13	338±65	n.s.
Liver	8	10.0 ± 0.7^{a}	13.7±2.6	*
	24	12.0 ± 1.0^{b}	15.1±2.8	*
	48	11.4 ± 0.8^{b}	14.3±3.0	n.s.
Relative liver weight (%)	8	3.2 ± 0.3^{a}	4.1 ± 0.3^{a}	***
	24	3.7 ± 0.3^{b}	4.7 ± 0.3^{b}	***
	48	$3.4\pm0.2^{a,b}$	4.2 ± 0.2^{a}	***

Values expressed as Mean±SD. * $p<0.05$, *** $p<0.001$ *vs.* control. Single a, b, c unlike symbols indicate statistical significance ($p<0.05$) between two groups in the vertical plane for a particular measurement by unpaired t-test after significant ($p<0.05$) one-way ANOVA.

Liver weights were increased by cholesterol feeding reaching statistical significance in rats appraised at 8 and 24 h after the radioactive oral dose. Dietary cholesterol enrichment also increased relative liver weight which was evident at all three time points following the administration of radioactive lipids.

The importance of using an intubation familiarisation protocol was borne out by the observation that two animals bit through the feeding tube during the sham protocol. This did not occur with the radioactive oral dose.

6.3.1.2 Microsomal cholesterol and phospholipid contents

As in the two preceding chapters, microsomal cholesterol content was increased by cholesterol supplementation (Table 6.2). This reached statistical significance at all three time points following the administration of the radioactive dose.

As expected, no significant effect of cholesterol supplementation was detected on microsomal total phospholipid content (Table 6.2).

Table 6.2 *Microsomal cholesterol and phospholipid contents and Δ6-desaturase activity of rats fed a control or cholesterol enriched diet for 56 days at 8, 24 and 48 h after administration of radioactive fatty acids*

Measurement	Time (h)	Control	Chol.	Significance
Cholesterol ξ	8	77±7[a]	107±8[a]	***
	24	61±4[b]	79±6[b]	***
	48	56±7[b]	78±8[b]	***
Phospholipid ξ	8	449±41	492±37	n.s.
	24	450±21	462±31	n.s.
	48	456±25	471±25	n.s.
Cholesterol/phospholipid ψ	8	0.17±0.02[a]	0.22±0.02[a]	**
	24	0.14±0.02[b]	0.17±0.02[b]	**
	48	0.12±0.02[b]	0.17±0.02[b]	**
Δ6-desaturase activity ζ	8	533±92[a,b]	666±201	n.s.
	24	469±46[a]	546±50	*
	48	584±82[b]	592±19	n.s.

Values expressed as Mean±SD. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs.* control. ξ nmol/mg protein, ψ mol/mol, ζ Δ6-desaturase activity estimated with 0.5 mg microsomal protein (activity expressed as pmol/min/mg). Single a, b, c unlike symbols indicate statistical significance ($p<0.05$) between two groups in the vertical plane for a particular measurement by unpaired t-test after significant ($p<0.05$) one-way ANOVA.

The cholesterol-phospholipid ratio was increased by cholesterol supplementation (Table 6.2). Interestingly, the ratio was highest in both control and cholesterol fed groups 8 h after the administration of the radioactive dose. Thereafter, the ratio

generally diminished by the 48 h time point to a level similar to that observed in the previous cholesterol feeding experiments (Chapters 4 and 5). This possibly reflected to some extent the effect of the 21 h fast and subsequent refeeding on liver microsomal cholesterol content.

6.3.1.3 Microsomal Δ6-desaturase activity

Δ6-desaturase activity was generally higher in cholesterol supplemented rats (Table 6.2). Owing to some large variations in activities, however, this was only significantly evident at the 24 h time point ($p=0.022$).

6.3.1.4 Microsomal phospholipid fatty acid composition

Many cholesterol-induced alterations to microsomal phospholipid fatty acid composition were observed. The results presented are limited to fatty acids relevant to Δ9- and Δ6-desaturation (Table 6.3). They are expressed as nmol rather than relative amounts to eliminate the effects of differences of other fatty acids[1].

Table 6.3 *Microsomal total phospholipid fatty acid composition of rats fed a control or cholesterol enriched diet for 56 days* §

Fatty acid species	Control	Chol.	Significance
PUFA (n-6)			
18:2	94±7	120±7	***
18:3 #	1.9±0.2	1.6±0.4	n.s.
20:3	18±2	22±3	*
20:4	205±16	188±15	n.s.
22:4	2.4±0.3	1.7±0.2	**
22:5	8.4±2.0	4.7±1.3	**
Σ n-6	331±21	339±23	n.s.
Σ SFA	406±23	376±16	*
Σ MUFA	100±7	150±11	***

Values expressed as nmol/mg microsomal protein (Mean±SD). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs.* control. # Unresolved peak contained unspecified amounts of 20:0. § All measurements made 48 h after administration of radioactive fatty acids.

[1] To simplify presentation, only the fatty acid profile of rats sacrificed at 48 h is presented as this *generally* reflected the situation found at both 8 and 24 h time points. In addition, data on liver weights (Section 6.3.3.1) suggests that the rats are achieving re-equilibrium 48 h after their 21 h fast.

In line with the previous cholesterol feeding studies, 18:2 and 20:3 were significantly elevated by cholesterol supplementation. Similarly, cholesterol feeding generally reduced levels of 20:4, 22:4 and 22:5. Interestingly, 18:3 was elevated in cholesterol fed rats 8 h after the administration of radioactive fatty acids (0.9±0.1 *vs.* 1.1±0.1 nmol/mg microsomal protein, Table E.4, Appendix E) but no statistical difference was evident at the two subsequent time points analysed.

Cholesterol supplemented rats had higher levels of MUFA compared with controls (due to increased levels of 16:1, 18:1 and 20:1). This was accompanied by reduced levels of SFA (Table 6.3).

6.3.1.5 Microsomal phospholipid fatty acid ratios

The 18:3 n-6/18:2 n-6 Δ6-desaturase index did not significantly differ between cholesterol and control fed rats at the earliest time point after dosing with radioactivity but at the two subsequent time points the index was decreased in cholesterol fed rats. In contrast, the 22:5 n-6/22:4 n-6 Δ6-desaturase index was not influenced by cholesterol feeding (Table 6.4).

The 20:4 n-6/20:3 n-6 Δ5-desaturase index was at its peak level, in both groups, 8 h after the administration of radioactive fatty acids. At this time, the index in cholesterol fed rats was 50 % that of controls. This large difference between control and cholesterol supplemented rats was reduced at both 24 and 48 h after oral dosing with radioactivity, but the suppressive effect of cholesterol on this index remained significant.

The 18:2 D&E index was at its highest level in both groups 8 h after the administration of radioactive lipids. The index was lower in both groups at 24 h and recovered slightly by 48 h. Cholesterol feeding caused a significant decrease in this index at all three time points analysed.

Table 6.4 *Microsomal phospholipid fatty acid ratios of rats fed a control or cholesterol enriched diet for 56 days at 8, 24 and 48 h after administration of radioactive fatty acids*

Fatty acid ratio	Time (h)	Control	Chol.	Significance
n-6				
18:3/18:2 (Δ6)	8	0.010 ± 0.002^{a}	0.010 ± 0.001^{a}	n.s.
	24	0.032 ± 0.005^{b}	0.021 ± 0.002^{b}	**
	48	0.020 ± 0.003^{c}	0.014 ± 0.004^{a}	**
20:4/20:3 (Δ5)	8	27.1 ± 4.1^{a}	14.7 ± 2.4^{a}	***
	24	12.7 ± 0.9^{b}	8.6 ± 0.6^{b}	***
	48	11.3 ± 1.0^{c}	8.7 ± 0.7^{b}	***
22:5/22:4 (Δ6)	8	1.8 ± 0.2^{a}	1.7 ± 0.8^{a}	n.s.
	24	2.5 ± 0.7^{a}	$2.1\pm0.5^{a,b}$	n.s.
	48	3.4 ± 0.5^{b}	2.8 ± 0.7^{b}	n.s.
18:2 D&E	8	2.9 ± 0.2^{a}	2.1 ± 0.3^{a}	***
	24	2.3 ± 0.1^{b}	1.6 ± 0.0^{b}	***
	48	2.5 ± 0.2^{b}	1.8 ± 0.1^{a}	***
n-9				
18:1/18:0 (Δ9)	8	0.32 ± 0.03^{a}	0.42 ± 0.03^{a}	***
	24	0.47 ± 0.03^{b}	0.71 ± 0.05^{b}	***
	48	0.43 ± 0.04^{b}	0.74 ± 0.08^{b}	***

Values expressed as Mean±SD. ** $p<0.01$, *** $p<0.001$ *vs.* control. Single a, b, c unlike symbols indicate statistical significance ($p<0.05$) between two groups in the vertical plane for a particular measurement by unpaired t-test after significant ($p<0.05$) one-way ANOVA. 18:2 D (Desaturation) & E (Elongation) refers to (18:3 n-6 + 20:2 n-6 + 20:3 n-6 + 20:4 n-6 + 22:4 n-6 + 22:5 n-6)/18:2 n-6.

Cholesterol supplementation increased the Δ9-desaturation index (18:1/18:0 ratio) at each of the three time points following the administration of the radioactive dose (Table 6.4).

6.3.1.6 Plasma lipid content and composition

Cholesterol fed rats had slightly higher levels of plasma total cholesterol compared to controls. This reached statistical significance 48 h after the administration of radioactive lipid (Table 6.5). Cholesterol ester concentrations generally reflected the results demonstrated for the total cholesterol levels.

No significant differences were apparent for triacylglycerol levels between cholesterol and control fed rats. Plasma phospholipid concentrations were also not significantly different between the two groups at each of the three time points after oral dosing with radioactivity.

Table 6.5 *Plasma total cholesterol, cholesterol ester, phospholipid and triacylglycerol concentrations and phospholipid and cholesterol ester fatty acid compositions of rats fed a control or cholesterol enriched diet for 56 days* §

Plasma lipid and fatty acid species	Control	Chol.	Significance
Total cholesterol ‡	2.0±0.3	3.6±0.9	**
Triacylglycerol ‡	1.7±0.5	1.4±0.4	n.s.
Phospholipid ‡	1.5±0.2	1.5±0.2	n.s.
PUFA (n-6)			
18:2	594±93	625±104	n.s.
20:2	2.9±0.9	3.5±1.1	n.s.
18:3 #	5.1±1.3	5.0±1.0	n.s.
20:3	65±10	69±10	n.s.
20:4	451±65	377±28	*
22:4	7.3±0.9	5.0±0.6	***
22:5	21±6	11±3	*
Σ n-6	1146±166	1096±133	n.s.
Σ SFA	1346±204	1172±161	n.s.
Σ MUFA	453±87	585±102	*
Cholesterol ester ‡	1.7±0.3	2.7±0.5	**
PUFA (n-6)			
18:2	399±53	381±54	n.s.
18:3 #	19±2	15±4	n.s.
20:3	22±6	20±3	n.s.
20:4	753±144	448±60	**
22:4	0.9±1.0	0.8±1.0	n.s.
22:5	2.6±1.4	1.2±0.8	n.s.
Σ n-6	1196±199	865±106	**
Σ SFA	143±20	368±95	**
Σ MUFA	262±37	1428±452	**

Fatty acids expressed as μmol/l (Mean±SD). ‡ Expressed as mmol/l. * $p<0.05$, ** $p<0.01$ *** $p<0.001$ *vs.* control. # Unresolved peak contained unspecified amounts of 20:0. § All measurements made 48 h after administration of radioactive fatty acids.

As in the previous cholesterol feeding experiment, marked changes were discernible in the fatty acid profile of the phospholipid fraction[2]. Cholesterol feeding significantly increased the amount of MUFA in plasma phospholipids (levels of 16:1, 18:1 and 20:1 were elevated by 57, 26 and 40 %, respectively). However, total levels of SFA in plasma phospholipids were unaffected by cholesterol supplementation despite a small but significant decrease in 18:0. The contribution of n-6 fatty acids with two or three double bonds in plasma phospholipids was not significantly altered by

[2] To simplify presentation, only the fatty acid profile of rats sacrificed at 48 h is presented as this *generally* reflected the situation found at both the 8 and 24 h time points.

cholesterol supplementation. Nevertheless, levels of 20:4, 22:4 and 22:5 were significantly reduced in cholesterol fed rats (Table 6.5).

Despite cholesterol ester levels being approximately 50 % higher in rats receiving cholesterol, the total amount of n-6 fatty acids was significantly decreased (Table 6.5). A 40 % reduction in 20:4 was mainly responsible for this finding. Of interest, levels of 18:2, 18:3, 20:3, 22:4 and 22:5 did not alter as a result of the cholesterol supplementation. Total levels of SFA were highly increased as a result of the cholesterol feeding. (16:0 was increased two-fold and 18:0 increased five-fold). Similarly, total amounts of MUFA were markedly increased (16:1 was increased four-fold while both 18:1 and 20:1 were increased six-fold).

Only minor cholesterol induced differences in total plasma ^{14}C and ^{3}H were detected (Appendix E). The majority of plasma ^{14}C was incorporated into phospholipids with slightly smaller amounts present in cholesterol esters. The identity of the radioactive fatty acids in plasma lipid fractions was not determined and these plasma radioactivity results will not be discussed further.

6.3.2 Microsomal radioactive fatty acids

6.3.2.1 Microsomal phospholipid [^{14}C]labelled fatty acids

There was a time related decrease in the total amount of ^{14}C present in microsomal phospholipids. Samples obtained 8 h post intubation contained approximately five times as much ^{14}C as those collected at 48 h (Table 6.6). Total ^{14}C in microsomal phospholipids did not differ significantly between cholesterol and control fed rats at each of the three time points assessed.

At both 8 and 24 h after the oral administration of radioactive fatty acids, [^{14}C]-diene and [^{14}C]triene fatty acid counts were not different between cholesterol and control fed rats. At the same time points, however, [^{14}C]tetraene counts were significantly reduced by dietary cholesterol enrichment (Table 6.6). By the final time point (48 h) more differences between the two groups were evident: levels of

[^{14}C]diene fatty acids were significantly increased while [^{14}C]tetraenes were significantly reduced by the cholesterol supplementation.

Table 6.6 *Distribution of ^{14}C in microsomal phospholipid fatty acids of rats fed a control or cholesterol enriched diet for 56 days at 8, 24 and 48 h after administration of radioactive fatty acids*

Label	Time (h)	Control	Chol.	Significance
[^{14}C]phospholipid #	8	3945±304^{a}	3258±1068^{a}	n.s.
	24	1282±225^{b}	1438±398^{b}	n.s.
	48	651±137^{c}	711±113^{c}	n.s.
[^{14}C]dienes	8	3330±288^{a}	2826±862^{a}	n.s.
	24	796±159^{b}	1080±279^{b}	n.s.
	48	216±62^{c}	349±65^{c}	**
[^{14}C]trienes	8	236±29^{a}	206±67^{a}	n.s.
	24	189±38^{b}	188±53^{a}	n.s.
	48	87±18^{c}	103±33^{b}	n.s.
[^{14}C]tetraenes	8	268±53	103±43^{a}	***
	24	264±31	130±60^{a}	**
	48	335±69	235±33^{b}	*
[^{14}C]SFA & [^{14}C]MUFA	8	111±48^{a}	123±124	n.s.
	24	33±13^{b}	41±21	n.s.
	48	14±5^{c}	25±12	n.s.

Values expressed as DPM/mg microsomal protein (Mean±SD). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs.* control. # Total ^{14}C in the phospholipid fraction was calculated by the adding together ^{14}C present in diene, triene, tetraene, monoene and saturated fatty acids. Single a, b, c unlike symbols indicate statistical significance ($p<0.05$) between two groups in the vertical plane for a particular measurement by unpaired t-test after significant ($p<0.05$) one-way ANOVA.

The TLC band that comprised SFA and MUFA also contained small amounts of ^{14}C (Table 6.6). Slightly higher counts of these fatty acids were observed in microsomal phospholipids of rats supplemented with cholesterol. The difference, however, did not reach statistical significance.

6.3.2.2 Specific activity of [^{14}C]labelled microsomal phospholipid fatty acids

Specific activities of n-6 fatty acids extracted from microsomal phospholipids are shown in Table 6.7.

Table 6.7 *Specific activity of microsomal phospholipid [^{14}C]labelled n-6 fatty acids of rats fed a control or cholesterol enriched diet for 56 days at 8, 24 and 48 h after administration of [1-^{14}C]18:2 n-6*

Measurement	Time (h)	Control	Chol.	Significance
N-6 diene specific activity	8	38.9±4.4^{a}	24.0±7.3^{a}	**
	24	8.0±1.7^{b}	8.3±1.9^{b}	n.s.
	48	2.3±0.7^{c}	2.9±0.5^{c}	n.s.
N-6 triene specific activity	8	25.4±5.2^{a}	12.7±4.6^{a}	**
	24	10.1±2.1^{b}	8.2±2.2^{a}	n.s.
	48	4.3±0.9^{c}	4.4±1.2^{b}	n.s.
N-6 tetraene specific activity	8	1.2±0.2^{a}	0.5±0.2^{a}	***
	24	1.3±0.2^{a}	0.7±0.3^{a}	**
	48	1.6±0.3^{b}	1.2±0.2^{b}	*

Values expressed as DPM/nmol (Mean±SD). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs.* control. For dienes the denominator is 18:2 n-6 plus 20:2 n-6 (<1.0 nmol 20:2 n-6 per mg microsomal protein), for trienes the denominator is 18:3 n-6 plus 20:3 n-6 and for tetraenes the denominator is 20:4 n-6 plus 22:4 n-6. Single a, b, c unlike symbols indicate statistical significance ($p<0.05$) between two groups in the vertical plane for a particular measurement by unpaired t-test after significant ($p<0.05$) one-way ANOVA.

N-6 diene specific activity decreased with time after oral dosing with values at 48 h approximately 1/17th and 1/8th of those existing at 8 h in control and cholesterol fed rats, respectively. A significantly lower n-6 diene specific activity was observed in cholesterol supplemented rats at 8 h post intubation. A similar finding was observed for n-6 triene specific activity. Specific activities of n-6 tetraene fatty acids on the other hand were lower in cholesterol supplemented rats at the 8, 24 and 48 h time points (Table 6.7).

6.3.2.3 Microsomal phospholipid [^{3}H]labelled fatty acids

Count rates for ^{3}H decreased with time from the oral administration of radioactivity (Table 6.8). Samples obtained 8 h post intubation contained approximately six times as much ^{3}H as those collected at 48 h.

Table 6.8 *Distribution of ^{3}H in microsomal phospholipid fatty acids of rats fed a control or cholesterol enriched diet for 56 days at 8, 24 and 48 h after administration of [11,12-^{3}H]18:0*

Measurement	Time (h)	Control	Chol.	Significance
[^{3}H]phospholipid	8	2799±476^a	2088±592^a	*
	24	870±322^b	911±273^b	n.s.
	48	345±191^c	364±110^c	n.s.
[^{3}H]SFA	8	2799±476^a	2063±592^a	*
	24	826±309^b	829±248^b	n.s.
	48	329±180^c	327±99^c	n.s.
[^{3}H]MUFA	8	n.d.	24±11^a	-
	24	44±15^a	82±28^b	*
	48	17±12^b	38±14^a	*

Values expressed as DPM/mg microsomal protein (Mean±SD). * $p<0.05$ *vs.* control. n.d. Not detectable. - Not statistically tested. Single a, b, c unlike symbols indicate statistical significance ($p<0.05$) between two groups in the vertical plane for a particular measurement by unpaired t-test after significant ($p<0.05$) one-way ANOVA.

Eight h after the administration of [11,12-^{3}H]18:0, cholesterol fed animals had significantly higher levels of ^{3}H in microsomal phospholipids compared with controls. No significant difference was observed between the two groups at the two later time points. Levels of ^{3}H in SFA showed the same pattern as that described for ^{3}H incorporation into total phospholipid fatty acids (Table 6.8).

Incorporation of ^{3}H in MUFA was highly elevated by cholesterol supplementation. At each of the three time points after the administration of [11,12-^{3}H]18:0 cholesterol fed animals had increased counts in the monoene fraction. This reached statistical significance 48 h post intubation and supports the view that cholesterol feeding has induced Δ9-desaturase. Direct measurement of Δ9-desaturase activity in one half of the animals was increased (50±30 pmol/min/mg) in cholesterol fed rats compared with controls (23±13 pmol/min/mg, $p=0.03$). However, the activity of quality control stock

material had decreased during storage and the same was probably true for all samples. Thus, Δ9-desaturase activity in this study may only be an indication and absolute levels may be quite different. Other microsomal Δ9-desaturase activities were not measured because of this very low activity.

6.3.2.4 Specific activity of [^{3}H]labelled microsomal phospholipid fatty acids

The specific activities of SFA and MUFA are shown in Table 6.9. Eight h after the administration of radioactivity, cholesterol fed rats had lower a SFA specific activity than controls. This difference was not apparent at the two subsequent time points.

Table 6.9 *Specific activity of microsomal phospholipid [^{3}H]labelled fatty acids of rats fed a control or cholesterol enriched diet for 56 days at 8, 24 and 48 h after administration of [11,12-^{3}H]18:0*

Measurement	Time (h)	Control	Chol.	Significance
SFA specific activity	8	6.7±1.1^a	4.7±1.3^a	*
	24	2.1±0.7^b	2.2±0.6^b	n.s.
	48	0.8±0.4^c	0.9±0.3^c	n.s.
MUFA specific activity	8	n.d.	0.2±0.1^a	-
	24	0.4±0.1^a	0.5±0.1^b	n.s.
	48	0.1±0.1^b	0.2±0.1^a	n.s.

Values expressed as DPM/nmol (Mean±SD). * $p<0.05$ *vs.* control. For SFA specific activity the denominator is total microsomal SFA, for MUFA specific activity the denominator is total microsomal MUFA. Single a, b, c unlike symbols indicate statistical significance ($p<0.05$) between two groups in the vertical plane for a particular measurement by unpaired t-test after significant ($p<0.05$) one-way ANOVA. - Not statistically tested.

Monoene specific activity was slightly higher in cholesterol fed rats at all time points after the oral administration of radioactivity. This, however, did not reach statistical significance.

6.3.2.5 Microsomal phospholipid specific activity ratios

Specific activity ratios derived from n-6 PUFA, MUFA and SFA data are presented in Table 6.10. Cholesterol supplementation significantly reduced the triene/diene and 18:2 D&E specific activity ratios at each time point following the administration of radioactive fatty acids. The depressive effect of cholesterol feeding on the tetraene/triene ratio was only significantly evident at the 24 h time point.

Table 6.10 *Indices of [1-^{14}C]18:2 n-6 and [11,12-^{3}H]18:0 metabolism derived from microsomal phospholipid fatty acid specific activities of rats fed a control or cholesterol enriched diet for 56 days at 8, 24 and 48 h after administration of radioactive fatty acids*

Measurement	Time (h)	Control	Chol.	Significance
^{14}C				
Triene SA/Diene SA #	8	0.65±0.07	0.52±0.07	*
	24	1.26±0.12	0.98±0.10	**
	48	1.95±0.26	1.49±0.20	**
^{14}C				
Tetraene SA/Triene SA #	8	0.05±0.01	0.04±0.01	n.s.
	24	0.13±0.02	0.09±0.02	**
	48	0.38±0.05	0.31±0.12	n.s.
^{14}C				
18:2 D&E SA index #	8	0.68±0.07	0.54±0.07	**
	24	1.42±0.14	1.06±0.12	***
	48	2.68±0.42	1.93±0.17	**
^{3}H				
MUFA SA/SFA SA #	8	n.d.	0.04±0.03	-
	24	0.18±0.04	0.21±0.02	n.s.
	48	0.17±0.08	0.25±0.05	n.s.

Values expressed as Mean±SD. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. 18:2 D&E SA index relates to (triene SA + tetraene SA)/diene SA. # SA specific activity. - Not statistically tested.

Cholesterol supplementation only tended to increased the MUFA/SFA specific activity ratio ($p=0.080$ and $p=0.067$ at the 24 and 48 h time points, respectively; Table 6.10).

6.3.3 Adipose tissue fatty acid composition and radioactivity

6.3.3.1 Adipose tissue triacylglycerol fatty acid composition

The effects of cholesterol supplementation on adipose tissue fatty acid composition were minor. There was a small but significant decrease in the percentage of 16:0 while 18:1 and 18:2 n-6 were both increased (Table 6.11).

Table 6.11 *Adipose tissue triacylglycerol fatty acid composition of rats fed a control or cholesterol enriched diet for 56 days* §

Fatty acid species	Control	Chol.	Significance
SFA			
14:0	2.2±0.1	2.1±0.1	n.s.
16:0	22.6±0.5	21.6±0.5	**
18:0	11.2±1.5	11.2±1.4	n.s.
20:0 #	0.1±0.0	0.1±0.0	n.s.
MUFA			
16:1	4.4±0.7	3.9±0.6	n.s.
18:1	47.3±0.7	48.3±0.6	*
20:1	1.1±0.0	1.2±0.1	n.s.
PUFA (n-6)			
18:2	9.4±0.4	9.9±0.3	*
20:4	0.1±0.0	0.1±0.0	n.s.
PUFA (n-3)			
18:3	0.3±0.0	0.3±0.0	n.s.

Values expressed as % (w/w) total fatty acids (Mean±SD). * $p<0.05$, ** $p<0.01$ *vs.* control. # Unresolved peak contained unspecified amounts of 18:3 n-6. § All measurements made 48 h after administration of radioactive fatty acids.

6.3.3.2 Adipose tissue [1-^{14}C]linoleic acid and metabolites

No ^{14}C was detected in fatty acids other than dienes in adipose tissue (Table 6.12). Cholesterol supplemented rats had slightly higher levels of [1-^{14}C]dienes than controls but owing to large variability the difference was not significant. No difference in the amount of n-6 dienes present in adipose tissue was manifest and no difference in the specific activity of this fatty acid was observed.

Table 6.12 *Adipose tissue triacylglycerol diene content, radioactivity and specific activity of rats fed a control or cholesterol enriched diet for 56 days* §

Measurement	Control	Chol.	Significance
[^{14}C]diene ψ	4000±2100	5400±2000	n.s.
Diene content ξ	2160±280	2160±300	n.s.
Diene specific activity	1.9±1.0	2.4±0.6	n.s.

Values expressed as Mean±SD. ψ DPM/10 mg adipose tissue, ξ nmol/10 mg adipose tissue. § All measurements made 48 h after administration of radioactive fatty acids.

6.3.3.3 Adipose tissue [11,12-^{3}H] stearic acid and metabolites

Adipose tissue SFA and MUFA ^{3}H results are presented in Table 6.13. There was large variability in the level of ^{3}H in both fractions and no significant difference in the amount of ^{3}H in SFA and MUFA was apparent. The specific activities of these respective fractions were slightly higher in cholesterol fed rats but this did not achieve statistical significance.

Table 6.13 *Adipose tissue triacylglycerol [11,12-^{3}H]18:0 metabolism of rats fed a control or cholesterol enriched diet for 56 days* §

Measurement	Control	Chol.	Significance
[^{3}H]SFA ψ	1190±630	1570±610	n.s.
SFA content ξ	8828±1151	8159±1301	n.s.
SFA specific activity #	13.4±7.3	18.7±5.2	n.s.
[^{3}H]MUFA ψ	310±160	440±170	n.s.
MUFA content ξ	12102±1335	11638±1691	n.s.
MUFA specific activity #	2.6±1.4	3.7±1.0	n.s.

Values expressed as Mean±SD. ψ DPM/10 mg adipose tissue, ξ nmol/10 mg adipose tissue, # Specific activities multiplied by 100. § All measurements made 48 h after administration of radioactive fatty acids.

6.4 Discussion

The central objective of this study was to use an alternative method to elucidate the effect of cholesterol supplementation on the metabolism of 18:2 n-6. This *in vivo* approach, carried out along similar lines to a study conducted by Zevenbergen and Houtsmuller (1989), concentrated upon [1-^{14}C]18:2 n-6 metabolism in the liver microsomal fraction as this forms the main site of 18:2 n-6 Δ6-desaturation in rats. The metabolism of [11,12-^{3}H]18:0 by liver microsomal Δ9-desaturase was used as a comparison.

As anticipated cholesterol supplementation modified key parameters, such as microsomal cholesterol, fatty acid composition and Δ6-desaturase activity, in a manner generally consistent with the findings of the previous cholesterol feeding studies. Results of the radioactive fatty acids, on the other hand, depend on how the data is expressed. Using pure count rate data, there was no significant difference in the total amount of ^{14}C in microsomal phospholipids between cholesterol and control fed rats. However, the distribution of ^{14}C in dienes, trienes and tetraenes was distinctly altered. Despite some high count variability, it was clear that cholesterol feeding caused a marked decline in [^{14}C]tetraenes particularly 8 h after dosing with radioactive substrate. The expected accumulation of precursor ([^{14}C]dienes), however, was only manifest at the 48 h time point.

The specific activity results, a more usual method of expressing data from radioactive tracer studies, differed slightly from pure count rate data. No increase in n-6 diene specific activity was observed at the 48 h time point but then the initial n-6 diene specific activity was significantly lower in cholesterol fed rats at 8 h. Nevertheless, n-6 tetraene specific activity was markedly reduced at 8, 24 and 48 h.

The specific activity ratio pertinent to Δ6-desaturation was lower in cholesterol supplemented rats and the index of overall metabolism of 18:2 n-6 revealed a similar pattern. Therefore, combining all the data from this *in vivo* study it is evident that

cholesterol supplementation reduces the metabolism of 18:2 n-6 in rats in a manner consistent with a *reduced* liver microsomal Δ6-desaturase activity.

It is of interest that the conclusion of a comparable (but less comprehensive) experiment *in vivo*, studying the effects of *trans*-fatty acids on 18:2 n-6 metabolism, was different despite similar specific activity data (Zevenbergen and Houtsmuller, 1989). They found that tetraene specific activity was much higher in rats fed a diet containing olive oil, whereas that of dienes and trienes was hardly influenced by the dietary fats studied. They suggested that the diets examined did not affect 20:4 n-6 synthesis during the first steps of 18:2 n-6 conversion (i.e. Δ6-desaturation and elongation) but during the events that take place *after* the formation of 20:3 n-6 (i.e Δ5-desaturation). Yet, the specific activity of tetraene fatty acids must depend to some extent on the metabolism of 18:2 n-6 at the level of Δ6-desaturase and although the results of the present study are similar to those of Zevenbergen and Houtsmuller (1989) a different conclusion can be drawn.

The results of Δ6-desaturase activities measured *in vitro* form a distinct contrast to the data obtained *in vivo*. This difference may relate to the way in which the *in vivo* data is obtained and questions whether the conclusions of the study *in vivo* can be quite as definite. For example, the argentation TLC method used only separated fatty acids according to the degree of unsaturation. Thus each of the n-6 PUFA specific activities had to be calculated using a denominator that was the combined total of two n-6 fatty acids. The triene/diene specific activity ratio, for example, included 20:3 n-6 as well as 18:3 n-6 and as a result may not relate strictly to the activity of Δ6-desaturase *per se* but to Δ6-desaturation plus elongation. This methodological limitation may also have had important implications for the MUFA/SFA specific activity ratio. This ratio only showed a trend toward an increase in cholesterol fed rats but the microsomal Δ9-desaturase activity obtained *in vitro* was significantly increased by cholesterol feeding (despite a smaller group size).

Other limitations exist with the *in vivo* study data. At the 8 h time point, for example, microsomal phospholipid diene specific activity was significantly lower in cholesterol fed rats. This could indicate that some of the administered substrate had already been metabolised by Δ6-desaturase by this time point. Yet, other routes for substrate utilisation are available *in vivo*. Increased substrate oxidation in cholesterol fed rats may well have influenced diene specific activity. Indeed, this is supported by increased levels of [^{14}C]SFA and [^{14}C]MUFA in microsomal phospholipids of cholesterol fed rats, consistent with increased mitochondrial and peroxisomal β-oxidation of [1-^{14}C]18:2 n-6 with subsequent esterification of the *de novo* synthesised [^{14}C]fatty acids (Zevenbergen and Houtsmuller, 1989; Grønn *et al.*, 1992). Lower [1-^{14}C]18:2 n-6 absorption may also have affected microsomal diene specific activity but it is more likely that greater dilution of the substrate within the phospholipid 18:2 n-6 pool as well as other associated lipid compartments contributed to the lower specific activity of this diene fraction.

Δ6-desaturase activities were in some cases highly variable. They were adjusted for endogenous ^{14}C present in the microsomal lipid but even then the variability was greater than that presented in the previous cholesterol feeding studies (Chapters 4 and 5). The 21 h fast before the oral administration of radioactivity was used to facilitate radio-label uptake. Unfortunately, this resulted in rats being analysed at different degrees of *refeeding* which may have altered desaturase activities to different extents. Furthermore, fasting depresses Δ6-desaturase activity while refeeding reactivates the system (Brenner *et al.*, 1968; De Gómez Dumm *et al.*, 1970). The greatest variability in Δ6-desaturase activities was generally observed 8 h after the administration of radioactive fatty acids and may relate to individual responses to the 8 h period in which refeeding occurred. In addition, diurnal variations also alter desaturase activities (De Gómez Dumm *et al.*, 1984). The activity of Δ6-desaturase is higher at 2000 hours than at 1200 hours (De Gómez Dumm *et al.*, 1984), a pattern that was generally mirrored

by the results of this study. These influences on the activity of Δ6-desaturase should be considered carefully in the design of similar experiments *in vivo*.

Despite differences between *in vivo* and *in vitro* systems, the results for [11,12-^{3}H]18:0 metabolism by Δ9-desaturase *in vivo* were more clear cut. Although cholesterol feeding did not reduce [^{3}H]SFA (except at the 8 h time point), [^{3}H]MUFA levels were increased in cholesterol fed rats at all three time points following the administration of radioactive fatty acids. Using specific activities, the cholesterol feeding effect was reduced but still evident in the monoene fraction. These results were in agreement with microsomal Δ9-desaturase activity obtained *in vitro* (although these were only tentative results) as well as the microsomal Δ9-desaturase fatty acid index. Previous studies examining the effect of cholesterol feeding on Δ9-desaturase activity measured in microsomal preparations *in vitro* are also compatible with these results (Leikin and Brenner, 1987; Garg *et al.*, 1988a).

Incorporation of ^{14}C and ^{3}H was also demonstrated in adipose tissue triacylglycerols. Levels of ^{14}C were only measurable in the dienes with undetectable levels of ^{14}C in the more unsaturated n-6 fatty acids. No effect of cholesterol supplementation was observed. These results are thus inconclusive with respect to the metabolism of 18:2 n-6 to longer chain n-6 fatty acids and possibly relate to the short duration of the study but also to the original low level of long chain n-6 PUFA.

Detectable levels of ^{3}H in the monoene fraction were present in adipose tissue triacylglycerols. The origin of [^{3}H]MUFA may have arisen from the Δ9-desaturation of [11,12-^{3}H]18:0 *in situ* (Wahle, 1974) or from the circulating plasma triacylglycerol compartment. This study, however, did not differentiate between these two sources.

A number of study components were considered at the outset of this *in vivo* experiment. Not least was the form of the orally dosed radioactive fatty acids. The ingestion of triacylglycerols formed the basis of the dietary fat intake. However, it was not considered necessary to esterify radioactive fatty acids to glycerol as good label uptake was demonstrated in a pilot study with [1-^{14}C]18:2 n-6 and a similar fasting

protocol. The amount and type of carrier oil were also considered carefully. Oral dosing supplementation studies have been carried out with daily doses as high as 0.7 ml of dietary oil (Du, 1991). This present investigation used the oil purely as a carrier for the radioactive fatty acids. A dose of 0.2 ml was chosen as it permitted accurate volume administration. The type of dosing oil was also believed to be important. I used a highly purified olive oil which was low in 18:2 n-6 (approximately 13 % w/w) so that the [1-^{14}C]18:2 n-6 was not diluted excessively. The use of molten *dietary* fat, which was used as the oral dosing agent in some studies (Zevenbergen and Houtsmuller, 1989) was not deemed practical in the present study as it was largely saturated (m.p. ~34°C) and was not sufficiently liquid at temperatures easily employable for oral dosing.

Therefore in conclusion, this study demonstrated once more that Δ6-desaturase activity measured *in vitro* is increased by cholesterol supplementation. Much of the data *in vivo*, however, indicates that the metabolism of 18:2 n-6 is impaired by dietary cholesterol. Yet, since the two approaches measure similar but different facets of 18:2 n-6 metabolism these results can be rationalised.

Chapter 7

Linoleic acid metabolism in the diabetic BB/E rat

7.1 Introduction

Elevated amounts of 18:2 n-6 with depressed levels of 20:4 n-6 in plasma, serum, platelet and red blood cell lipids from Type 1 (insulin-dependent, ketosis prone) human diabetics have been generally regarded to reflect an impaired 18:2 n-6 metabolism (Jones *et al.*, 1983; van Doormaal *et al.*, 1988; Horrobin, 1992). However, not all diabetic studies have documented such fatty acid patterns (Kalofoutis and Lekakis, 1981; Tilvis and Miettinen, 1985; Freyberger *et al.*, 1989). Diet can also be a determinant of tissue fatty acid composition and it is noteworthy that this often differs between diabetics and the general population. Unfortunately, only a few human diabetic studies examine both diet and tissue fatty acid compositions. Indeed, Tilvis and Miettinen (1985) have documented that diabetic patients consume 40 % more 18:2 n-6 despite a total fat intake comparable with controls. In accord with this are World Health Organisation (WHO) recommendations that propose a reduction in saturated fat with a concomitant increase in unsaturates for diabetics (WHO, 1990). The American Diabetic Association also puts forward such dietary advice (WHO, 1990). This kind of dietary difference may skew diabetic tissue fatty acid compositions in a manner that could be interpreted incorrectly as a depressed Δ6-desaturase activity.

Diabetes leads to a number of serious secondary complications such as atherosclerotic cardiovascular disease due to macroangiopathy and microangiopathic complications such as retinopathy, nephropathy and neuropathy. One proposed factor implicated in the manifestation of these diabetic complications is an impaired 18:2 n-6 metabolism by Δ6-desaturase (Horrobin, 1993; Jamal, 1994). The resultant reduction in long chain n-6 fatty acids has been postulated to lead to abnormal erythrocyte, platelet, endothelial and neurone function. The question is whether diabetic patients influence tissue fatty acid composition by a higher dietary intake of 18:2 n-6 or

whether there is an impaired conversion of 18:2 n-6 to longer chain n-6 fatty acids. Measurements of liver microsomal Δ6-desaturase activity in diabetic humans are not generally feasible because of the ethical constraints associated with collection of liver biopsies. Fortunately, the spontaneously diabetic Bio Breeding (BB) rat provides a useful alternative with a destructive insulitis comparable with humans (Baird, 1989; Chanussot *et al.*, 1989; Elias *et al.*, 1990; Okamoto, 1990). The aim of the present study was to examine the effect of diabetes with and without normal insulin treatment on the level of the regulatory enzyme Δ6-desaturase. This was compared with the fatty acid compositions of liver, plasma and adipose tissue in rats fed a highly controlled diet.

7.2 Methods

Twenty-four, male, Wistar BB/Edinburgh (E) rats, consisting of twelve insulin-dependent diabetics and twelve normal controls, from the Edinburgh Western General Hospital colony, were used. All rats were fed a commercially available chow (Appendix F) that contained 2.5 % (w/w) fat with a fatty acid composition of 0.2 % 14:0, 17.2 % 16:0, 0.3 % 16:1, 1.7 % 18:0, 15.8 % 18:1, 57.4 % 18:2 n-6, 6.5 % 18:3 n-3 and 0.4 % 20:1. In the diabetic prone BB/E rat strain, spontaneous diabetes is expressed in 50-60 % of animals at approximately 90 d of age (detected by urinary glucose appearance and abnormal weight gain; Baird, 1989). In the control (sub-line) strain of BB/E rats less than 1 % of animals develop diabetes by the age of one year. When symptoms were detected, diabetic rats were maintained on daily subcutaneous insulin injections essential for survival. Insulin dose was evaluated for each individual rat, by highly experienced staff, to maintain body weight (monitored daily) and for normal metabolic requirements (absence of urinary glucose and ketone bodies).

Diabetic rats were divided into two equal groups, matched for age, duration of diabetes and insulin treatment (Table 7.1); one group continued the usual once daily insulin injection with tissues (blood, liver and adipose tissue) assessed 6 h after the last

insulin dose. The other group were given their routine insulin treatment and then deprived of insulin for 54 h (at which time tissues were assessed). For simplifying the description of the results, diabetic rats assessed 6 h post insulin injection will be referred to as 'insulin treated' and diabetic rats withheld from insulin for 54 h will be known as 'insulin withdrawn'.

Tissues from two BB/E, age-matched, non-diabetic, control groups were also analysed (Table 7.1). The original control group rats (assessed alongside the diabetic animals) were bred from a normal BB/E, non-diabetic pair whose family history at the time of study was negative with respect to diabetes. However, the male of the breeding pair later developed diabetes at seven months of age. The results of Δ6-desaturase activity measurements from this group were heterogeneous and therefore another BB/E, non-diabetic, age-matched control group was selected as a replacement. This substitute group will be referred to as the control but results from the 'abnormal' group will also be presented as a comparison (generally without results of statistical analysis).

Table 7.1 *Group description*

Measurement	Control (n=6)	Control † (n=6)	Diabetic Assessed 6 h post insulin treatment (n=6)	Diabetic Assessed 54 h post insulin treatment (n=6)
Age #	152±5	153±1	152±10	150±11
Duration of diabetes #	n.a.	n.a.	61±4	60±6
Insulin treatment ξ	n.a.	n.a.	3.0±0.3	2.7±0.1

Values expressed as Mean±SD. † 'Abnormal' control. # d, ξ IU/d. n.a. Not applicable. No statistically significant differences were observed.

Fed rats were anaesthetised between 1545 and 1615 hours with Sagatal® (60 mg/kg body weight, Rhône Mérieux, Dublin, Eire). Blood was withdrawn from the heart and plasma and red blood cells prepared as described in Sections 2.3.2 and 2.4.4, respectively. Rats were sacrificed by excising the heart (under anaesthesia) and then both liver and adipose tissue were removed and processed. The study was designed so

that liver microsomes were prepared from two animals from each group on three separate days. This was to facilitate rapid processing and to limit the influence of systematic variations in liver microsomal and other tissue preparation. Liver was rinsed in homogenisation buffer, wrapped in aluminium foil and surrounded with ice for immediate carriage to the laboratory (approximately 45 min). Liver microsomes were prepared as described (Section 2.3.1) and Δ6-desaturase activity measured under optimal conditions (Section 2.4.8). Microsomal and plasma lipid fatty acid compositions were determined as described in Sections 2.5.2 and 2.5.4, respectively. Plasma lipids, insulin and glucose were analysed as outlined in Sections 2.4.1, 2.4.2, and 2.4.3, respectively. Red blood cell glycosylated haemoglobin was determined as described in Section 2.4.4. Adipose tissue triacylglycerol fatty acid composition was analysed as described in Section 2.5.6.

7.3 Results

7.3.1 General animal characteristics

Body weight remained stable for the seven days preceding sacrifice in control and insulin treated diabetic rats. However, a profound body weight reduction was observed in diabetic rats which had insulin administration withdrawn (Fig. 7.1 and Table 7.2). At the time of insulin withdrawal (two days prior to sacrifice) weights were not significantly different from those at seven days before sacrifice. Yet, one day later and on the day of sacrifice, these diabetic rats had lost 15±12 g ($p=0.033$) and 30±14 g ($p=0.0035$), respectively. Unfortunately, under these conditions one animal died (which was subsequently replaced) and another had to be sacrificed early for humane reasons.

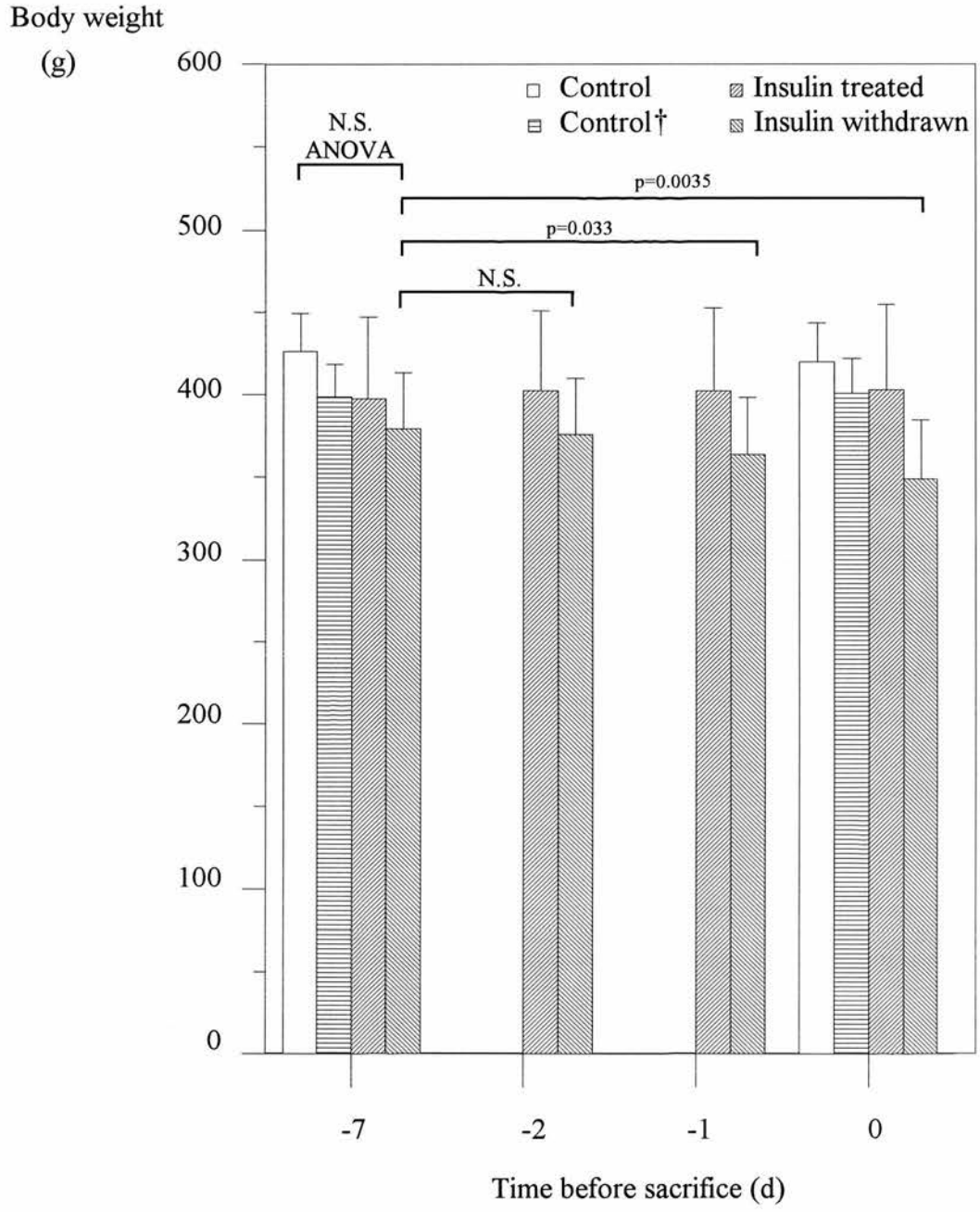


Figure 7.1 *Body weights of control and diabetic rats on the day of sacrifice and at 1, 2 and 7 days prior to sacrifice. Insulin withdrawn at -2 days. Results expressed as Mean±SD. Body weights were reduced after insulin withdrawal at -1 and 0 days ($p<0.05$, paired t-test).* † *Abnormal control.*

No significant differences were observed for the absolute weights of liver and heart. However, insulin treated diabetic animals had a significantly higher relative liver weight compared with controls and the insulin withdrawn group had a significantly raised relative heart weight (Table 7.2). The latter was the result of the acute body weight loss in this group.

Table 7.2 *Body, liver and heart weights of control and diabetic rats*

Measurement	Control (n=6)	Control † n=6	Diabetic Insulin treated (n=6)	Diabetic Insulin withdrawn (n=5)
Weight (g)				
Body	420±24^{x}	401±21	403±52^{x,y}	354±39^{y}
Liver	14.2±1.8	13.2±1.7	16.1±3.5	12.1±1.4
Heart	1.00±0.08	0.91±0.05	0.96±0.11	0.94±0.08
Relative weight (%)				
Liver/body	3.38±0.32^{x}	3.29±0.27	3.97±0.54^{y}	3.47±0.08^{x}
Heart/body	0.24±0.01^{x}	0.23±0.00	0.24±0.02^{x}	0.27±0.01^{y}

Values expressed as Mean±SD. All weights measured at sacrifice. Single x, y unlike symbols indicate statistical significance ($p<0.05$) between two groups in the horizontal plane for a particular measurement by unpaired t-test after significant ($p<0.05$) one-way ANOVA. † 'Abnormal' control.

7.3.2 Glycaemic control

Rats were in the fed state before sacrifice. Control animals demonstrated this with a moderate hyperglycaemia and hyperinsulinaemia (Table 7.3). Low plasma glucose levels were detected in insulin treated diabetic rats which were associated with insulin concentrations not dissimilar from control animals. Insulin withdrawn diabetic rats demonstrated a considerable hyperglycaemia, three-fold that of controls. Plasma insulin levels were consistent with this finding with three of the five samples below the level of detection (i.e. <2.5 mU/l, Table 7.3). The control group that had a family history of diabetes had plasma glucose and insulin levels not significantly different from the true controls.

Table 7.3 *General blood biochemistry of control and diabetic rats*

Measurement	Control (n=6)	Control † (n=6)	Diabetic Insulin treated (n=6)	Diabetic Insulin withdrawn (n=5)
Glucose ξ	9.0 ± 1.0^{x}	9.2±1.1	3.5 ± 1.4^{y}	28.6 ± 4.7^{z}
Insulin ψ	134 ± 42^{x}	125±79	173 ± 70^{x}	(5,5)#
GHb ζ	3.4 ± 0.1^{x}	3.0±0.3	6.1 ± 1.4^{y}	9.2 ± 1.9^{z}
Triacylglycerol ξ	n.d.	0.9±0.3	0.9 ± 0.4^{x}	14.7 ± 5.0^{y}
Cholesterol ester ξ	1.9 ± 0.2^{x}	1.9±0.2	2.0 ± 0.1^{x}	1.3 ± 0.2^{y}
Phospholipid ξ	1.6 ± 0.2^{x}	1.6±0.1	1.9 ± 0.2^{y}	3.5 ± 0.3^{z}

Values expressed as Mean±SD. n.d. Not determined. Single x, y, z unlike symbols indicate statistical significance ($p<0.05$) between two groups in the horizontal plane for a particular measurement by unpaired t-test after significant ($p<0.05$) one-way ANOVA. # n=2, three samples below level of detection. ξ mM, ψ mU/l, ζ %. † 'Abnormal' control.

The percentage of glycosylated haemoglobin (GHb), used as an indicator of glycaemic regulation, was doubled in treated diabetic rats compared with control animals ($p=0.006$). The metabolically compromised, insulin withdrawn diabetic animals showed a further 50 % increase in GHb levels ($p=0.002$ and $p=0.02$ *vs.* control and insulin treated diabetic rats, respectively). The percentage of GHb in the 'abnormal' control group was just significantly lower than controls ($p=0.045$).

7.3.3 Plasma lipids

Metabolic state also influenced plasma lipid concentrations. Compared with controls, treated diabetic rats had significantly higher plasma phospholipid levels ($p=0.014$) but cholesterol ester levels were not significantly different. Insulin withdrawal caused a large increase in both triacylglycerol and phospholipid levels, however cholesterol esters were decreased ($p=0.024$ *vs.* control).

7.3.4 Microsomal Δ6-desaturase activity

Despite slightly higher circulating levels of insulin, Δ6-desaturase activity was decreased 25 % in treated diabetic rats compared with controls (p=0.013, Table 7.4). In diabetic rats in whom insulin was withdrawn for 54 h, Δ6-desaturase activity was 39 % lower than controls (p=0.002). The difference between the two diabetic groups did not reach statistical significance (p=0.12, unpaired t-test).

Table 7.4 *Microsomal Δ6-desaturase activity, non-esterified 18:2 n-6 and phospholipid concentrations of control and diabetic rats*

Measurement	Control (n=6)	Control † (n=6)	Diabetic Insulin treated (n=6)	Diabetic Insulin withdrawn (n=5)
Δ6-desaturase activity ζ	367 ± 53^{x}	308±180	277 ± 48^{y}	223 ± 54^{y}
Non-esterified 18:2 n-6 #	1.9 ± 0.2^{x}	2.5±0.4	2.3 ± 1.2^{x}	7.7 ± 3.7^{y}
Phospholipid #	543 ± 17^{x}	473±35	482 ± 29^{y}	$544\pm57^{x,y}$

Values expressed as Mean±SD. Single x, y unlike symbols indicate statistical significance (p<0.05) between two groups in the horizontal plane for a particular measurement by unpaired t-test after significant (p<0.05) one-way ANOVA. # nmol/mg protein, ζ pmol/min/mg protein. † 'Abnormal' control.

The concentration of non-esterified 18:2 n-6 was not significantly different between control and insulin treated diabetic rats (Table 7.4). Insulin withdrawal caused a four-fold increase in microsomal non-esterified 18:2 n-6 in diabetic rats compared with control animals (p=0.024).

The activity of Δ6-desaturase in the 'abnormal' control group is worthy of comment. Within this group there appeared to be two distinct sub-groups of three rats. In the first, a very low activity was demonstrated (150±5 pmol/min/mg). In the other, a high activity was manifest (471±69 pmol/min/mg). Levels of non-esterified 18:2 n-6 were 2.8±0.6 and 2.2±0.2 nmol/mg microsomal protein, for these low and high activity sub-groups, respectively (p=0.22, unpaired t-test). This could therefore not explain this difference in Δ6-desaturase activity. In addition, microsomal phospholipid levels were not differently distributed among high and low activity groups. It had been assumed

that these 'abnormal' control rats were from normal parents. Measurements such as urinary glucose were not carried out as this measurement is only used for diabetic animals monitored to determine the onset of diabetes. This information is not routinely carried out for sub-line animals because they only rarely become diabetic.

7.3.5 Microsomal phospholipid content

Insulin treated diabetic rats had significantly lower levels of microsomal phospholipids (expressed as nmol/mg protein) compared with control animals ($p=0.0031$; Table 7.4). Diabetic rats in whom insulin was withdrawn had phospholipid levels comparable with controls.

Although generally statistical results are not shown for 'abnormal' controls, the phospholipid results are worthy of note. The content of microsomal phospholipids of the 'abnormal' control group was significantly lower than controls ($p=0.0033$) but not statistically significant from both diabetic groups.

7.3.6 Microsomal phospholipid fatty acid composition

Microsomal phospholipid fatty acid composition of diabetic rats differed in many respects in comparison to control animals (Table 7.5). Although total levels of SFA in insulin treated diabetic rats did not differ from control rats, the composition did. There was a significant increase in 18:0 and a reduction in 16:0. Insulin withdrawal for 54 h raised the total amount of SFA in microsomal phospholipids of diabetic rats. This was due mainly to 18:0 ($p=0.0013$ *vs.* control). MUFA content and composition remained virtually unchanged.

Table 7.5 *Liver microsomal total phospholipid fatty acid composition of control and diabetic rats*

Fatty acid species	Control (n=6)	Control † (n=6)	Diabetic Insulin treated (n=6)	Diabetic Insulin withdrawn (n=5)
SFA				
14:0	4.3±0.5	6.6±2.1	4.6±2.6	2.8±1.0
16:0	$257±8^{x}$	235±15	$224±16^{y}$	$232±35^{x,y}$
18:0	$182±10^{x}$	224±16	$223±34^{y}$	$335±42^{z}$
MUFA				
16:1	$20±3^{x}$	18±2	$26±6^{x}$	$13±3^{y}$
18:1	118±10	114±11	122±14	109±23
20:1	$3.7±0.3^{x}$	15±3	$9.2±4.3^{y}$	$13±4^{y}$
PUFA (n-6)				
18:2	$133±9^{x,y}$	112±11	$125±8^{x}$	$156±19^{y}$
20:2	$5.0±0.6^{x}$	5.7±1.6	$5.0±2.2^{x,y}$	$2.7±1.5^{y}$
18:3 #	$2.0±0.1^{x}$	3.2±1.0	$5.4±1.8^{y}$	$3.3±2.1^{x,y}$
20:3	$12±1^{x}$	8.6±1.3	$14±3^{x}$	$6.8±1.9^{y}$
20:4	$265±12^{x}$	163±15	$160±20^{y}$	$167±19^{y}$
22:4	$2.6±0.4^{x}$	1.9±0.3	$1.5±0.3^{y}$	$1.2±0.1^{y}$
22:5	$3.0±0.2^{x}$	2.2±0.6	$1.3±0.7^{y}$	$0.9±0.2^{y}$
PUFA (n-3)				
18:3	$1.0±0.1^{x}$	n.d.	$0.9±0.3^{x}$	n.d.
20:5	$2.4±0.2^{x}$	1.2±0.5	$3.6±1.8^{x}$	$1.6±0.4^{y}$
22:5	$8.2±1.0^{x}$	4.2±0.6	$4.5±0.9^{y}$	$3.7±0.7^{y}$
22:6	$66±4^{x}$	30±3	$33±6^{y}$	$39±8^{y}$
PUFA (n-9)				
20:3	n.d.	n.d.	1.7±1.3 § ‡	0.5, 1.7 ‡
Σ SFA	$444±17^{x}$	466±28	$451±44^{x}$	$570±75^{y}$
Σ MUFA	142±12	147±15	157±12	135±24
Σ n-6	$423±19^{x}$	297±29	$313±24^{y}$	$338±38^{y}$
Σ n-3	$78±4^{x}$	36±4	$42±5^{y}$	$44±8^{y}$
DBI	$197±3^{x}$	147±5	$152±5^{y}$	$140±8^{z}$

Values expressed as nmol/mg protein (Mean±SD). Single x, y, z unlike symbols indicate statistical significance ($p<0.05$) between two groups in the horizontal plane for a particular measurement by unpaired t-test after significant ($p<0.05$) one-way ANOVA. # Unresolved peak contained unspecified amounts of 20:0. n.d. not detected. † 'Abnormal' control. § n=3. ‡ Three samples below level of detection.

Insulin treated diabetic rats had levels of 18:2 n-6, 20:2 n-6 and 20:3 n-6 not dissimilar from control rats. However, 18:3 n-6 was significantly increased and 20:4 n-6, 22:4 n-6 and 22:5 n-6 were significantly lower than in control rats.

Insulin withdrawal had no significant effect upon the concentrations of either 18:2 n-6 or 18:3 n-6 but the remaining n-6 fatty acids were all significantly lower (Table 7.5). Small differences between the two diabetic groups existed: 18:2 n-6 was higher in the insulin withdrawn diabetic group ($p=0.019$), and the opposite was true for 20:3 n-6 ($p=0.0007$).

Total amounts of n-3 PUFA were significantly reduced in diabetic rats, irrespective of their insulin status, when compared with control. Insulin treated diabetic rats had lower levels of both 22:5 and 22:6 while in the insulin withdrawn group all n-3 fatty acids quantified were reduced (Table 7.5).

The DBI was also markedly affected. The index (calculated from the percentage (w/w) fatty acids) was significantly reduced in both diabetic groups compared with control animals (both $p<0.001$). Treated diabetic rats had a higher DBI compared to insulin withdrawn diabetic animals ($p=0.023$).

The animals from the 'abnormal' control group presented a fatty acid profile more compatible with the diabetic rats than with the true control group. This was demonstrated with total levels of both n-6 and n-3 PUFA (Table 7.5). In addition, 20:4 n-6, either presented as an absolute amount (163±15 nmol/mg) or as a percentage of total phospholipid fatty acids (17.2±0.6 %) was significantly lower than that demonstrated for controls (265±12 nmol/mg ($p<0.001$) and 24.4±0.5 % ($p<0.001$), respectively).

7.3.7 Microsomal phospholipid fatty acid ratios

Indices derived from the fatty acid composition of phospholipids are shown in Table 7.6. The 18:3 n-6/18:2 n-6 Δ6-desaturase index was elevated in the insulin treated diabetic group ($p=0.0076$ *vs.* control). However, insulin withdrawal reduced this index to a value no longer significantly different from control. The effect of diabetes on the Δ5-desaturase index differed from that on the index for Δ6-desaturase. A decrease in the Δ5-desaturase index was observed in insulin treated diabetic rats ($p<0.0001$ *vs.* control) but it was no longer significantly different in diabetic rats in whom insulin was withdrawn.

Table 7.6 *Fatty acid indices derived from microsomal phospholipid fatty acid ratios of control and diabetic rats*

Index	Control (n=6)	Control † (n=6)	Diabetic Insulin treated (n=6)	Diabetic Insulin withdrawn (n=5)
n-6				
18:3/18:2 (Δ6)	0.015 ± 0.002^{x}	0.028 ± 0.007	0.044 ± 0.016^{y}	0.021 ± 0.014^{x}
20:4/20:3 (Δ5)	21.3 ± 0.7^{x}	19.1 ± 1.7	11.5 ± 1.6^{y}	25.5 ± 4.9^{x}
22:5/22:4 (Δ6)	1.2 ± 0.1^{x}	1.1 ± 0.2	$0.9\pm0.3^{x,y}$	0.8 ± 0.1^{y}
18:2 D&E	2.18 ± 0.14^{x}	1.64 ± 0.07	1.51 ± 0.25^{y}	1.17 ± 0.14^{z}
n-9				
18:1/18:0 (Δ9)	0.65 ± 0.08^{x}	0.51 ± 0.03	0.56 ± 0.13^{x}	0.33 ± 0.07^{y}

Values expressed as Mean±SD. Single x, y, z unlike symbols indicate statistical significance ($p<0.05$) between two groups in the horizontal plane for a particular measurement by unpaired t-test after significant ($p<0.05$) one-way ANOVA. 18:2 D (Desaturation) & E (Elongation) refers to (18:3 n-6 + 20:2 n-6 + 20:3 n-6 + 20:4 n-6 + 22:4 n-6 + 22:5 n-6)/18:2 n-6. † 'Abnormal' control. No triene/tetraene ratio presented as on control rats had no detectable 20:3 n-9.

The 18:2 D&E index was highest in controls, significantly lower in treated diabetic animals and lower still in insulin withdrawal group. Both the 22:5 n-6/22:4 n-6 Δ6- and Δ9-desaturase indices demonstrated a similar pattern.

7.3.8 Plasma phospholipid fatty acid composition

Total amounts of SFA and MUFA in plasma phospholipids were significantly raised in both diabetic groups compared with control animals (Table 7.7). This was due mainly to increases in 18:0 and 18:1 in the respective fractions.

Table 7.7 *Plasma phospholipid fatty acid composition of control and diabetic rats*

Fatty acid species	Control (n=6)	Control † (n=6)	Diabetic Insulin treated (n=6)	Diabetic Insulin withdrawn (n=5)
SFA				
14:0	11±2	10±1	9.4±1.9	8.5±2.9
16:0	819±81^{x}	795±63	887±75^{x}	1615±113^{y}
18:0	494±53^{x}	515±39	678±90^{y}	1565±149^{z}
MUFA				
16:1	51±10	39±5	61±9	71±27
18:1	300±47^{x}	279±28	393±50^{y}	582±154^{z}
20:1	12±1^{x}	12±1	14±3^{x,y}	15±1^{y}
PUFA (n-6)				
18:2	587±41^{x}	541±49	795±107^{y}	1528±128^{z}
20:2	11±2^{x}	13±2	13±4^{x}	6.2±0.9^{y}
18:3 #	5.8±1.3	6.4±0.3	7.4±1.5	8.9±3.2
20:3	35±5^{x}	28±3	53±9^{y}	27±2^{z}
20:4	661±94^{x}	721±69	695±95^{x}	1268±117^{y}
22:4	12±1^{x}	12±2	8.6±1.5^{y}	8.0±2.2^{y}
22:5	9.3±1.4^{x}	13±2	5.9±2.1^{y}	4.2±2.0^{y}
PUFA (n-3)				
18:3	3.3±0.3^{x}	3.1±0.7	4.2±1.0^{x}	7.0±1.6^{y}
20:5	7.0±1.2	4.0±1.4	10.9±4.4	7.0±2.4
22:5	19±2^{x,y}	19±1	17±2^{x}	20±1^{y}
22:6	141±26^{x}	131±12	125±23^{x}	269±35^{y}
Σ SFA	1324±135^{x}	1319±102	1575±149^{y}	3188±244^{z}
Σ MUFA	363±58^{x}	330±34	468±55^{y}	668±181^{y}
Σ n-6	1320±136^{x}	1334±122	1578±151^{y}	2851±149^{z}
Σ n-3	170±28^{x}	156±15	157±24^{x}	303±35^{y}
18:2 D&E index	1.25±0.13^{x}	1.47±0.06	1.00±0.21^{y}	0.87±0.12^{y}

Values expressed as μmol/l (Mean±SD). Single x, y, z unlike symbols indicate statistical significance ($p<0.05$) between two groups in the horizontal plane for a particular measurement by unpaired t-test after significant ($p<0.05$) one-way ANOVA. 18:2 D (Desaturation) & E (Elongation) refers to (18:3 n-6 + 20:2 n-6 + 20:3 n-6 + 20:4 n-6 + 22:4 n-6 + 22:5 n-6)/18:2 n-6. # Unresolved peak contained unspecified amounts of 20:0. † 'Abnormal' control. 20:3 n-9 not detectable.

Significant alterations were also observed for n-6 PUFA (Table 7.7). Levels of 18:2 and 20:3 were increased in insulin treated diabetic rats while 22:4 and 22:5 were reduced when compared with controls. Insulin withdrawn diabetic animals showed

additional modifications: 18:2 and 20:4 were increased while 20:2, 20:3, 22:4 and 22:5 were decreased.

In the insulin withdrawal group, the presentation of plasma phospholipid fatty acids as absolute amounts conceals some important changes. This is because the total plasma phospholipid content in these animals was double that of controls. When the data is presented as relative amounts, 18:2 n-6 was increased in insulin withdrawn diabetic rats, 21.8±1.8 % *vs.* 18.6±1.3 % (p=0.01); and 20:4 n-6 was decreased compared with controls (18.1±1.6 % *vs.* 20.7±0.8 %, p=0.02).

7.3.9 Plasma cholesterol ester fatty acid composition

Widespread significant changes were also observed for plasma cholesterol ester fatty acids (Table 7.8).

The total amount of n-6 fatty acids remained the same for insulin treated diabetic rats as controls. However, the distribution therein was modified with 18:2, 18:3 and 20:3 all significantly elevated and 22:5 reduced. The total amount of n-3 fatty acids also remained similar to controls but 20:5 was doubled and 22:6 almost halved.

Insulin withdrawal reduced the levels of both n-6 and n-3 fatty acids in plasma cholesterol esters when compared with insulin treated diabetic rats. In particular, 20:4 n-6 was roughly halved. For the n-3 series, both 20:5 and 22:6 were significantly reduced by insulin withdrawal.

Table 7.8 *Plasma cholesterol ester fatty acid composition of control and diabetic rats*

Fatty acid species	Control (n=6)	Control † (n=6)	Diabetic Insulin treated (n=6)	Diabetic Insulin withdrawn (n=5)
SFA				
14:0	6.5±0.9	7.5±1.5	5.9±1.1	4.3±2.6
16:0	$176±18^{X}$	183±16	$167±10^{X}$	$145±4^{Y}$
18:0	$9.5±1.8^{X}$	25±3	$13±3^{X}$	$43±11^{Y}$
MUFA				
16:1	$85±17^{X}$	66±11	$112±12^{Y}$	$43±14^{Z}$
18:1	$105±13^{X}$	102±14	$145±18^{Y}$	$137±25^{Y}$
20:1	$6.1±4.1^{X}$	15±6	$5.7±1.4^{X}$	$21±7^{Y}$
PUFA (n-6)				
18:2	$387±25^{X}$	363±30	$457±19^{Y}$	$317±65^{X}$
20:2	n.d.	n.d.	n.d.	n.d.
18:3 #	$14±1^{X}$	16±2	$20±4^{Y}$	$11±1^{Z}$
20:3	$8.9±1.0^{X}$	7.1±0.4	$12±2^{Y}$	$2.8±0.9^{Z}$
20:4	$1062±126^{X}$	1094±96	$1007±133^{X}$	$591±131^{Y}$
22:4	$0.6±0.1^{X}$	0.7±0.4	$0.5±0.2^{X}$	$2.1±1.0^{Y}$
22:5	$0.4±0.1^{X}$	0.9±0.3	$0.2±0.2^{Y}$	n.d.
PUFA (n-3)				
18:3	n.d.	n.d.	n.d.	n.d.
20:5	$15±2^{X}$	6.2±1.3	$28±8^{Y}$	$4.8±2.8^{Z}$
22:5	0.6±0.2	0.6±0.1	0.5±0.2	0.4±0.5
22:6	$33±4^{X}$	22±3	$22±4^{Y}$	$12±3^{Z}$
Σ SFA	192±21	215±20	185±13	193±15
Σ MUFA	$196±28^{X}$	183±25	$263±18^{Y}$	$201±40^{X}$
Σ n-6	$1472±146^{X}$	1482±127	$1497±132^{X}$	$923±182^{Y}$
Σ n-3	$49±6^{X}$	29±4	$50±10^{X}$	$17±6^{Y}$
18:2 D&E index	$2.80±0.26^{X}$	3.08±0.08	$2.28±0.30^{Y}$	$1.94±0.34^{Y}$

Values expressed as μmol/l (Mean±SD). Single x, y, z unlike symbols indicate statistical significance ($p<0.05$) between two groups in the horizontal plane for a particular measurement by unpaired t-test after significant ($p<0.05$) one-way ANOVA. 18:2 D (Desaturation) & E (Elongation) refers to (18:3 n-6 + 20:2 n-6 + 20:3 n-6 + 20:4 n-6 + 22:4 n-6 + 22:5 n-6)/18:2 n-6. # Unresolved peak contained unspecified amounts of 20:0. † 'Abnormal' control. 20:3 n-9 not detectable.

The cholesterol ester fatty acid derived 18:2 D&E index reflected the Δ6-desaturase activities measured in liver microsomes. This index was highest in controls and lowest in insulin withdrawn diabetic animals. However, no significant difference was found between the two diabetic groups ($p=0.12$).

7.3.10 Adipose tissue triacylglycerol fatty acid composition

Diabetic rats showed significantly lower levels of n-6 and n-3 PUFA in adipose tissue compared with control animals. The reduction in these PUFA was associated with approximately a 30 % increase in MUFA and a small rise in SFA (Table 7.9).

The large fall in n-6 PUFA in diabetic rats was due mainly to significant reductions in 18:2, 20:4, 22:4 and 22:5. Percentages of 20:2 and 20:3 (both n-6) were not significantly different from controls. Analysis of individual n-3 fatty acids revealed that the depletion in the total percentage of this class was due to significant decreases in all n-3 fatty acids. Despite the rise in MUFA in both groups of diabetic animals, the large reductions in PUFA resulted in a significantly reduced DBI compared with control.

Table 7.9 *Adipose tissue triacylglycerol fatty acid composition of control and diabetic rats*

Fatty acid species	Control (n=6)	Control † (n=6)	Diabetic Insulin treated (n=6)	Diabetic Insulin withdrawn (n=5)
SFA				
14:0	1.2 ± 0.1^{X}	1.4±0.1	1.6 ± 0.2^{Y}	1.5 ± 0.2^{Y}
16:0	24.1±0.7	22.6±0.7	26.0±1.6	24.5±1.8
17:0	0.3 ± 0.0^{X}	0.4±0.1	0.3 ± 0.0^{Y}	0.5 ± 0.0^{Z}
18:0	2.5 ± 0.1^{X}	2.6±0.1	2.9 ± 0.2^{Y}	3.4 ± 0.4^{Y}
20:0 #	0.2 ± 0.0^{X}	0.4±0.2	0.3 ± 0.1^{Y}	0.5 ± 0.1^{Y}
MUFA				
16:1	5.0 ± 0.4^{X}	5.1±0.6	9.7 ± 1.4^{Y}	$7.8\pm2.1^{X,Y}$
18:1	25.3 ± 0.5^{X}	25.7±0.5	29.6 ± 1.2^{Y}	29.2 ± 2.4^{Y}
20:1	0.7 ± 0.1^{X}	1.0±0.3	0.8 ± 0.1^{Y}	1.1 ± 0.4^{Y}
PUFA (n-6)				
18:2	35.8 ± 1.1^{X}	35.9±1.7	25.4 ± 2.9^{Y}	28.1 ± 4.6^{Y}
20:2	0.3±0.0	0.4±0.2	0.3±0.1	0.3±0.2
20:3	0.1±0.0	0.2±0.1	0.2±0.1	0.2±0.1
20:4	0.7 ± 0.1^{X}	0.7±0.1	0.4 ± 0.1^{Y}	0.3 ± 0.1^{Y}
22:4	0.2 ± 0.0^{X}	0.2±0.0	0.1 ± 0.0^{Y}	0.0 ± 0.0^{Y}
22:5	0.1 ± 0.0^{X}	0.1±0.0	0.0 ± 0.0^{Y}	0.0 ± 0.0^{Y}
PUFA (n-3)				
18:3	2.5 ± 0.1^{X}	2.5±0.2	1.7 ± 0.2^{Y}	1.8 ± 0.1^{Y}
20:5	0.1 ± 0.0^{X}	0.0±0.0	0.0 ± 0.0^{Y}	0.0 ± 0.0^{Y}
22:5	0.2 ± 0.0^{X}	0.3±0.4	0.1 ± 0.0^{Y}	0.0 ± 0.0^{Y}
22:6	0.4 ± 0.1^{X}	0.2±0.1	0.1 ± 0.1^{Y}	0.1 ± 0.1^{Y}
PUFA (n-9)				
20:3	n.d.	n.d.	n.d.	n.d.
Unknown	0.5 ± 0.0^{X}	0.4±0.0	0.5 ± 0.1^{X}	0.5 ± 0.1^{X}
Σ SFA	28.2 ± 0.8^{X}	27.4±0.6	31.1 ± 1.6^{Y}	$30.3\pm1.5^{X,Y}$
Σ MUFA	31.0 ± 0.4^{X}	31.8±1.1	40.2 ± 2.4^{Y}	38.2 ± 3.9^{Y}
Σ n-6	37.2 ± 1.1^{X}	37.5±1.4	26.2 ± 3.1^{Y}	29.0 ± 4.7^{Y}
Σ n-3	3.1 ± 0.3^{X}	2.8±0.3	2.0 ± 0.3^{Y}	2.0 ± 0.2^{Y}
DBI	82 ± 1^{X}	83±2	75 ± 3^{Y}	75 ± 2^{Y}

Values expressed as % (w/w) of total fatty acids (Mean±SD). Single x, y, z unlike symbols indicate statistical significance ($p<0.05$) between two groups in the horizontal plane for a particular measurement by unpaired t-test after significant ($p<0.05$) one-way ANOVA. † 'Abnormal' control. n.d. Not detectable. # Unresolved peak contained unspecified amounts of 18:3 n-6.

7.3.11 Correlations of Δ6-desaturase activity and fatty acid indices

A highly significant correlation was observed between Δ6-desaturase activity and microsomal 18:2 D&E index (Fig. 7.2 and Table 7.10). The relationship was weaker but still significant for 18:2 D&E indices derived from two species of plasma lipids when correlated with Δ6-desaturase activity.

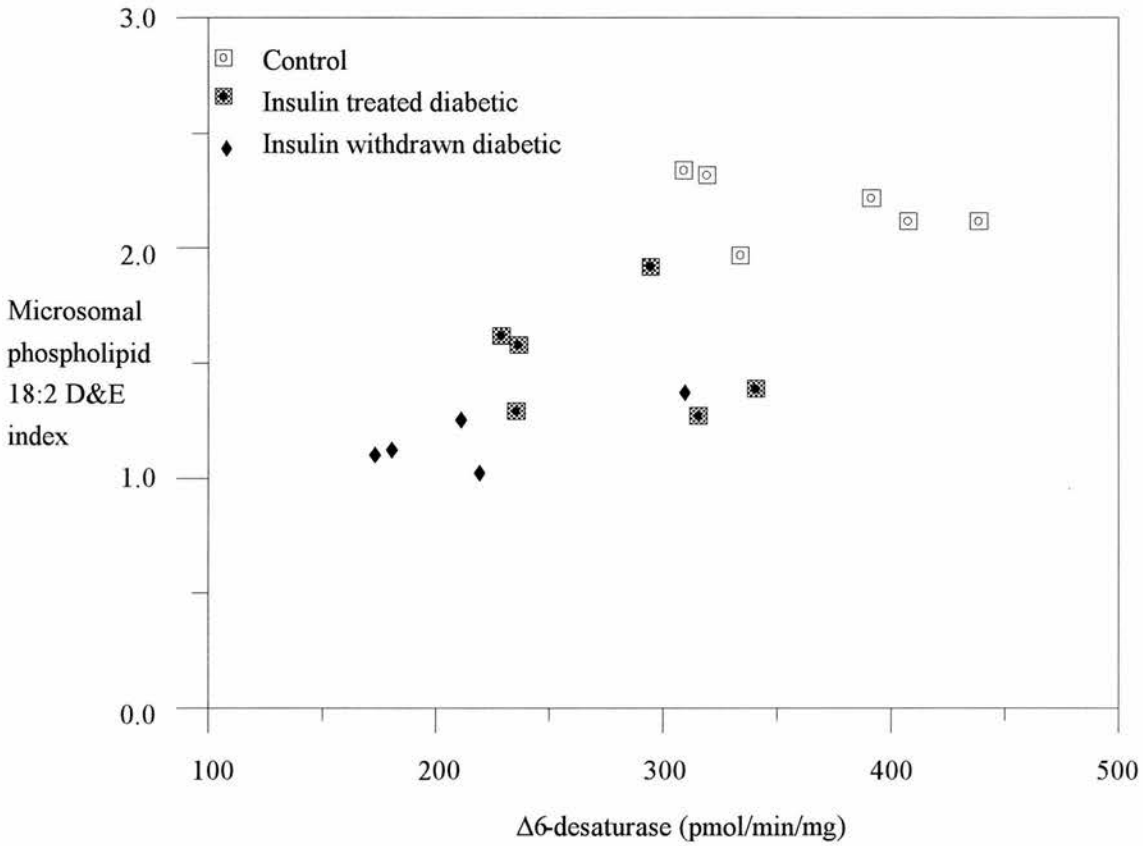


Figure 7.2 *Correlation of Δ6-desaturase activity and microsomal phospholipid 18:2 D&E index in control and diabetic rats*

Table 7.10 *Correlation matrix of Δ6-desaturase activity and tissue lipid fatty acid indices*

Measurement	Δ6-desat. activity	Mic. PL Δ6 (18:3/18:2)	Mic. PL Δ6 (22:5/22:4)	Mic. PL 18:2 D&E	Plasma PL 18:2 D&E
Δ6-desat. activity	--------	--------	--------	--------	--------
Mic. PL Δ6 (18:3/18:2) index	-0.179	--------	--------	--------	--------
Mic. PL Δ6 (22:5/22:4) index	0.652**	-0.230	--------	--------	--------
Mic. PL 18:2 D&E index	0.729***	-0.102	0.736***	--------	--------
Plasma PL 18:2 D&E index	0.545*	0.000	0.783***	0.888***	--------
Plasma CE 18:2 D&E index	0.526*	-0.128	0.598*	0.872***	0.869***

Values of *r* are derived from data from 17 animals (insulin treated and insulin withdrawn diabetic rats (n=11) and control rats (n=6)). * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Mic. microsomal, PL phospholipid, CE cholesterol ester, 18:2 D (Desaturation) & E (Elongation) refers to (18:3 n-6 + 20:2 n-6 + 20:3 n-6 + 20:4 n-6 + 22:4 n-6 + 22:5 n-6)/18:2 n-6.

All correlations with the 18:3 n-6/18:2 n-6 Δ6-desaturase index were not significant (Table 7.10). However, the 22:5 n-6/22:4 n-6 Δ6-desaturase index correlated well with Δ6-desaturase activity and the 18:2 D&E index from microsomal and plasma lipids.

7.4 Discussion

The BB diabetic rat is one of only two animal models available that are genetically susceptible to insulin-dependent diabetes. BB diabetic prone rats spontaneously develop autoimmune diabetes, characterised by destructive insulitis, that closely corresponds to the situation manifest in Type 1 human diabetics (Baird, 1989; Chanussot *et al.*, 1989; Elias *et al.*, 1990; Okamoto, 1990). The incidence of the disease in the BB/E diabetic prone population is 50-60 % by the age of 90-100 d. The model is relatively new and has not been studied extensively with respect to EFA metabolism. Other diabetic models have been concerned with the use of toxic diabetogenic agents such as streptozotocin (Rakieten *et al.*, 1963) and alloxan (Dunn *et al.*, 1943) which produce a form of diabetes that does not fully mirror the disease presented in humans. These toxic agents also have important side-effects on liver (Marliss *et al.*, 1982; Okamoto, 1990) which is particularly relevant when assessing a hepatic based enzyme system such as Δ6-desaturase. Previous diabetic studies from one laboratory group, examining Δ6-desaturase activity in BB rat colonies, have been contradictory (Chanussot *et al.*, 1989; Mimouni and Poisson, 1990; Mimouni and Poisson, 1992). Unsuitable control groups (e.g. the use of normal Wistar rats instead of BB Wistar) combined with inadequate assay conditions for Δ6-desaturase estimation employed in these studies, may have led to some of these anomalies and inconsistencies.

The results described also differed depending on which control group was employed. It is essential that a control group closely parallels the experimental model. For the BB/E diabetic rat, a non-diabetic, diabetes resistant, control is used preferentially over the normal Wistar strain rats as they are closer linked genetically. The origin of the diabetic and control animals in the study is shown in Figure 7.3.

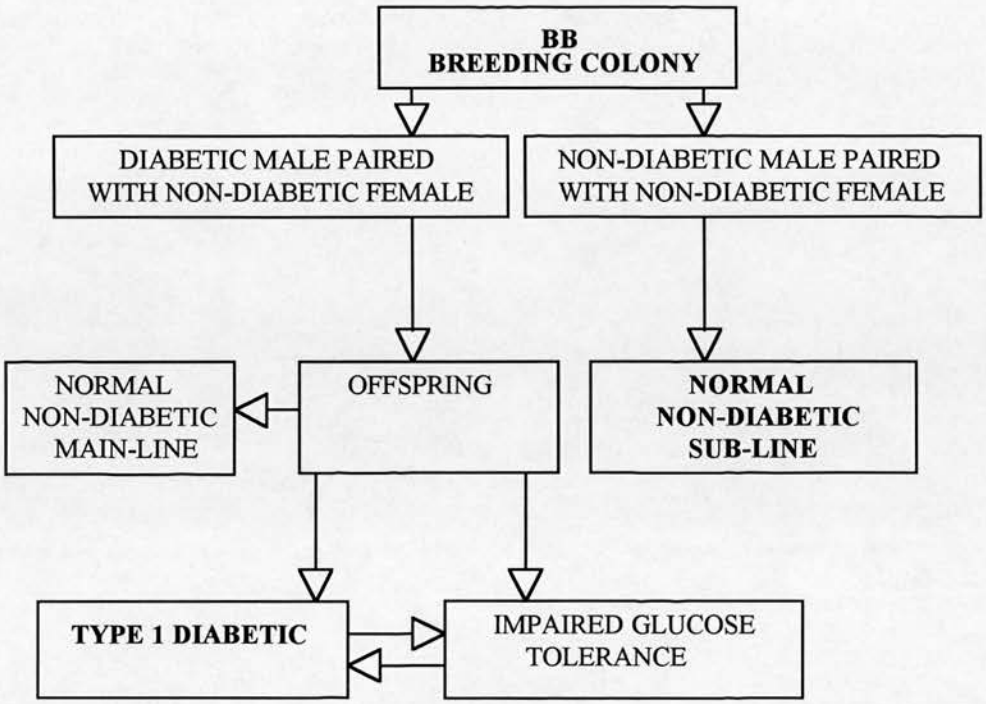


Figure 7.3 *Control and diabetic group origins (Control animals selected from the normal non-diabetic sub-line as normal non-diabetic main-line rats respond in a variable manner to oral glucose tolerance tests (Baird, 1989))*

The original control group, derived from a breeding pair of non-diabetic animals, revealed Δ6-desaturase activities that were distinctly heterogeneous. Subsequent family history examination showed that diabetes had been present in the father of the litter. Unfortunately, this finding was not known at the time of study and therefore liver, plasma and urine analyses were not available to give a more detailed description. The family history of the replacement control group, also non-diabetic sub-line, was checked thoroughly before being studied. This second group were fed the same diet and demonstrated adipose tissue 18:2 n-6 levels, a good marker for 18:2 n-6 intake (Wood *et al.*, 1987; Katan *et al.*, 1991), compatible with the original 'abnormal' control group. The use of this new control group therefore seems justified. In retrospect, however, some way of prior testing the control group such as a glucose tolerance test may have highlighted the 'abnormal' control group earlier. Unfortunately, this was not part of the project licence and therefore could not be carried out at that time.

As explained above, Δ6-desaturase activity has been examined in BB diabetic rats but the published results have been contradictory. This present study re-examined the effect of diabetes and employed a thoroughly characterised Δ6-desaturase method with a control group that was as close as possible to the diabetic line used. Δ6-desaturase activity was reduced by 25 % in the treated diabetic rats, while those that had undergone insulin withdrawal had a Δ6-desaturase activity 39 % lower than controls. The findings of this study and other studies on the diabetic BB rat are shown in Table 7.11.

Table 7.11 *Comparison of studies on Δ6-desaturase activity and liver microsomal fatty acid composition in the BB diabetic rat*

Study	Chanussot *et al.* (1989)	Mimouni and Poisson (1990)	Mimouni and Poisson (1992)	Brown *et al.* (1993)
BB rat characteristics	Orléans strain, female, 21 weeks old, 4 weeks, diabetic duration	Orléans strain, female, 16 weeks old, 3 weeks diabetic duration	Orléans strain, female, 16 weeks old, 7 weeks diabetic duration	Edinburgh strain, male, 22 weeks old, 9 weeks diabetic duration
n/group	5	3	4	6
Time of analysis	Diab. rats analysed 20 h after last insulin injection	Diab. rats analysed 3, 17 and 48 h after last insulin injection	Diab. rats given insulin dose to achieve hypo-, normo- and hyper-glycaemic states	Diab. rats analysed 6 and 54 h after last insulin injection
Control rat characteristics	Normal Wistar, female rats, 21 weeks old	Normal Wistar, female rats, 16 weeks old	Non-diabetic, Wistar BB, female rats, 16 weeks old	Non-diabetic, Wistar BB, male rats, 22 weeks old
Assay conditions	5 mg mic. protein, 40 nmol 18:2 n-6, 5 min incubation.	5 mg mic. protein, 40 nmol 18:2 n-6, 5 min incubation	5 mg mic. protein, 40 nmol 18:2 n-6, 5 min incubation	0.5 mg mic. protein, 200 nmol 18:2 n-6, 20 min incubation
Δ6-desaturase activity ζ	No significant difference. Control: 43.2±16.0 Diab.: 52.8±24.0	Control: 50.0±3.6 Diab. (3 h): 49.4±7.5 Diab. (17 h): 29.5±0.8 Diab. (48 h): 17.8±1.1	Control: 80.0±8.0 Hypo- diab.: 84.8±2.2 Normo- diab.: 49.6±6.4 Hyper- diab.: 33.6±9.6	Control: 367±53 Diab. (6 h): 277±48 Diab. (54 h): 223±54
Microsomal lipid composition	Total lipid: Increased 18:2 n-6 and decreased 20:4 n-6 in diab. rats	Total lipid: No significant difference between control and diab. rats in 18:2 n-6 and 20:4 n-6	Total lipid: Increased 18:2 n-6 in hyper-glycaemic state, decreased 20:4 n-6 in both normo- and hyper-glycaemic states	Total phospholipid: Decreased 20:4 n-6 in insulin treated diab. Increased 18:2 n-6 and decreased 20:4 n-6 in insulin withdrawn diab. rats.

Results expressed as Mean±SD. ζ pmol/min/mg microsomal protein. Mic. microsomal, diab. diabetic. Further details in text below.

The first paper comparing BB/Orléans (O) diabetic rats with controls (age and sex matched, normal *Wistar* rats) examined liver microsomal Δ6-desaturase activity, 20 h

after the last insulin injection (Chanussot *et al.*, 1989). Results demonstrated that Δ6-desaturase activity was not significantly different from controls at this time point and this was in contrast to the results of the microsomal *total* lipid n-6 fatty acid profile that suggested a reduced Δ6-desaturase activity (Table 7.11). A subsequent study by the same group (Mimouni and Poisson, 1990), examined liver microsomal Δ6-desaturase activity in BB/O diabetic rats at three different time points after insulin injection (Table 7.11). This study, using very small numbers (n=3/group), showed decreased Δ6-desaturase activity in diabetic rats 17 and 48 h post insulin injection (41 and 64 % less than in controls, respectively) but not at 3 h. *Wistar* controls were again used and the microsomal *total* lipid n-6 fatty acid composition was not significantly altered. Of particular interest are the diabetic rats analysed 48 h after the last insulin injection. These rats were in a severe hyperglycaemia (29.1±10.4 mM) which was similar to that demonstrated in the present study 54 h after insulin injection (28.6±4.7 mM). High levels of microsomal non-esterified 18:2 n-6 were demonstrated in our diabetic rats 54 h after insulin and so it would not be unreasonable to expect similarly high values of the same fatty acid in their diabetic rat microsomes 48 h after insulin injection. The authors, Mimouni and Poisson (1990), used 5 mg microsomal protein with 40 nmoles [1-^{14}C]18:2 n-6 for their Δ6-desaturase estimation. Their control microsomes were relatively low in activity (50.0±3.6 pmol/min/mg) and this may be due to some extent to protein substrate binding. More importantly, with such *high* amounts of microsomal protein combined with *low* substrate concentrations it becomes critical to correct for the dilution in substrate specific activity by endogenous non-esterified 18:2 n-6. If we assume that the non-esterified 18:2 n-6 content was ≅8 nmol/mg microsomal protein from diabetic rats, the specific activity of [1-^{14}C]18:2 n-6 in the assay would have been halved in comparison to non-diabetic controls. Therefore instead of a 64 % reduction in Δ6-desaturase activity, it would be 32 %. Mimouni and Poisson (1990) made no attempt to adjust for this endogenous non-esterified 18:2 n-6. Consequently, it is difficult to perceive their results are accurate and they may be

underestimating the effect of insulin withdrawal at this 48 h time point. Indeed, using the combination of low substrate and high microsomal protein concentrations provides the opportunity for control values of microsomal Δ6-desaturase to be likewise under-estimated by approximately 25 %. This by assuming the non-esterified 18:2 n-6 content as 2.0 nmol/mg microsomal protein, as documented in the present BB/E control group, but also in rats fed either cholesterol or control diet from Chapter 5. Nevertheless, these adjustments make no difference to the conclusion that insulin withdrawal for 48 h leads to a significant reduction in Δ6-desaturase activity.

The most recent work from Mimouni and Poisson (1992) used a different approach to determine the effect of insulin upon Δ6-desaturase activity in the BB diabetic rat. Their study aimed at establishing hyper-, normo- and hypo-glycaemic states in three groups of BB/O diabetic rats (Table 7.11). The hypoglycaemic phase (0.4±0.2 mM) induced by giving 10 IU insulin (per kg body weight) for two consecutive days prior to sacrifice, normalised Δ6-desaturase activity. Furthermore, much of the microsomal *total* lipid n-6 fatty acid profile showed a similar normalisation. Our study was designed so that Δ6-desaturase activity was analysed 6 h after the last insulin injection. This time point was chosen to coincide with peak protein synthesis rates. The activity of Δ6-desaturase, however, was 25 % lower than that present in control rats and it is apparent from other data of Mimouni and Poisson (1990) that normalisation of Δ6-desaturase occurs much sooner (i.e. 3 h post insulin injection; assuming low amounts of microsomal non-esterified 18:2 n-6).

Besides the methodological aspects of Δ6-desaturase measurement, the type of microsomal lipid analysed for fatty acid composition is also of importance (Table 7.11). Under normal conditions, microsomes contain mainly phospholipids but triacylglycerols and cholesterol esters are also present in minor amounts (Garg *et al.*, 1988b). The contribution of the latter lipids depends on nutritional and hormonal state and this is particularly relevant in diabetes. For example, in uncontrolled diabetes liver triacylglycerol content is dramatically increased (Murthy and Shipp, 1979; Howard,

1987). Microsomal triacylglycerol fatty acid composition differs markedly from that of phospholipids and so any increase in the proportion of this lipid will affect the total lipid fatty acid profile, in particular 20:4 n-6. The fatty acid profile of total microsomal phospholipids may therefore not be particularly useful for estimation of desaturase activities under different levels of circulating insulin. The use of a defined lipid pool, such as phospholipids, may therefore be superior in monitoring the differences that occur.

As with the previous chapters, rats were provided diets with a fatty acid composition which was devoid of all n-6 fatty acids other than 18:2 n-6. This means that fatty acids derived from 18:2 n-6 in lipid pools, such as microsomal phospholipids, may be used as an indicator of 18:2 n-6 metabolism. The microsomal phospholipid 18:3 n-6/18:2 n-6 Δ6-desaturase ratio was increased in the insulin treated diabetic rats but not significantly different in the insulin withdrawn group. The other Δ6-desaturase ratio (22:5 n-6/22:4 n-6) was decreased in the insulin treated diabetics and lower still in the diabetics withdrawn from insulin. It is therefore apparent that the indices for Δ6-desaturase differ depending on which fatty acids are used. Values obtained for the *activity* of the enzyme relate well with the 22:5 n-6/22:4 n-6 ratio for Δ6-desaturase and the index for total 18:2 n-6 desaturation and elongation. Yet, the more specific ratio (18:3 n-6/18:2 n-6) bears no relationship with Δ6-desaturase activity and suggests that other factors are involved in the regulation of phospholipid fatty acyl composition as suggested by Poisson and Cunnane (1991).

Adipose tissue triacylglycerol fatty acid composition was markedly altered by diabetes almost regardless of insulin treatment for the two days prior to sacrifice. Both diabetic groups presented lower relative amounts of n-6 and n-3 fatty acids with compensatory increases in the saturated and monounsaturated acids. Thus, even though once daily insulin injections maintained body weight, the treatment failed to normalise adipose tissue fatty acid composition. Only a small number of studies specifically document adipose triacylglycerol fatty acid composition in diabetes under

controlled dietary conditions. Reports of human diabetic adipose tissue composition are limited and generally show that there are no differences between diabetic and normal subjects (Heffernan, 1964; Antonini, *et al.*, 1970) but of course these studies lack dietary control.

Data on animals have been only concerned with *experimentally* induced diabetes to date. Streptozotocin induced diabetic rats show some similarities with the BB/E diabetic rat (Huang *et al.*, 1984). These include elevated amounts of 18:1 and depressed levels of 20:4 n-6, 22:5 n-6, 20:5 n-6 and 22:6 n-3. However, adipose 18:2 n-6 was raised in contrast to the BB/E diabetic rat. Alloxan induced diabetes shows an even greater contrast (Brenner *et al.*, 1968). Here the relative amounts of 18:2 n-6 and 20:4 n-6 were raised and the monounsaturated and saturated components decreased in epididymal fat compared with controls. This may relate to site specific differences in adipose tissue composition (Calder *et al.*, 1992), but also the extra-pancreatic toxicity of the diabetogenic agent.

In summary, it is clear that the activity of Δ6-desaturase is depressed in the insulin treated BB diabetic rat and reduced fractionally further by insulin withdrawal. Microsomal phospholipid fatty acid composition is altered in a manner that overall reflects this reduction of 18:2 n-6 metabolism but the more *specific* index for Δ6-desaturase (18:3 n-6/18:2 n-6) shows no relationship with activity. Despite a normalisation of plasma lipid levels, the n-6 fatty acid profile is also indicative of an impaired 18:2 n-6 metabolism. This is evident in both phospholipids and cholesterol esters. Further still, adipose tissue triacylglycerol fatty acid composition is not normalised by long term insulin therapy despite maintenance of body weight. It is therefore apparent from combining the above results with observations from the current literature that insulin therapy given subcutaneously does not provide adequate control to normalise Δ6-desaturase and other parameters measured. Ideally, the situation to be attained is for insulin therapy to be relevant for metabolic need. A large dose of insulin (double that required to maintain body weight and plasma lipid content)

can possibly correct the deficiency in Δ6-desaturase experimentally but is associated with extreme hypoglycaemia (Mimouni and Poisson, 1992). It is also not clear how relevant this is to diabetic patients. Tighter control of blood glucose concentrations, with consequent lower HbA_1 levels, have been associated with lower progression rates of neuropathy in patients but a greater frequency of hypoglycaemic phases (Diabetes Control and Complications Trial Research Group, 1993). Whether such treatment results in an improved Δ6-desaturation of 18:2 n-6 remains to be seen. However, improved nerve function has been shown experimentally by bypassing Δ6-desaturase with γ-linolenic acid (Lockett and Tomlinson, 1992) and it may be more rational to supplement with this fatty acid in addition to achieving a reasonable control of glycaemia in diabetic patients. Beneficial effects of γ-linolenic acid supplementation on nerve conduction have been demonstrated in diabetic patients (Jamal *et al.*, 1986; Keen *et al.*, 1993). Therefore until the effects of insulin therapy on Δ6-desaturation in man are understood, direct supplementation with γ-linolenic acid may be the way forward to combat serious complications in the diabetic population.

Chapter 8
General discussion

The explanation for the high CHD mortality rates in Scotland and other analogous regions in northern latitudes is still not fully understood. Dietary factors, such as a low intake of 18:2 n-6 have been associated with the disease process (Logan *et al.*, 1978) but an impaired metabolism of 18:2 n-6 to 20:3 n-6 and 20:4 n-6 has also been suggested in case-control and cross-sectional studies of CHD (Wood *et al.*, 1984; Riemersma *et al.*, 1986; Abraham *et al.*, 1990). The Δ6-desaturation of 18:2 n-6, believed to be rate limiting step in the conversion of 18:2 n-6 to 20:4 n-6, is slow in humans (De Gómez Dumm *et al.*, 1975) and may be influenced by known CHD risk factors, such as raised serum cholesterol, stress and diabetes. In this thesis, the effects of a number of risk factors for CHD on the activity of liver microsomal Δ6-desaturase activity and tissue fatty acid composition were examined.

At the outset of the work a range of methodological aspects pertinent to the study of Δ6-desaturase were considered. Argentation TLC was employed to separate the substrate from the products of Δ6-desaturation. This approach, in combination with scintillation counting, provides greatest sensitivity but lacks the specificity of techniques such as GLC and HPLC which can separate 18:3 n-6 from 20:3 n-6. Nevertheless, these two products would have to be summated if analysed by GLC or HPLC to obtain total Δ6-desaturase activity. Of course using GLC or HPLC can provide information about the specific activity of 18:3 n-6 which would be necessary to interpret any 20:3 n-6 data. Since, malonyl-CoA is not routinely added to the Δ6-desaturase incubation mixture there is little formation of 20:3 n-6 anyway. Therefore with these provisos in mind the results using argentation TLC are valid.

Another concern using argentation TLC was the potential for the formation of oxidised substrate that could interfere with Δ6-desaturation measurement. Products originating from 18:2 n-6 oxidation have a reduced mobility during argentation TLC and may coincide with the 18:3 n-6 band. The assay routinely used in the work

described in this thesis contained antioxidants and there was no evidence that under the conditions employed (using BHT and argon during work up) that 18:2 n-6 was oxidised in blanks or by boiled microsomes.

Contamination of the microsomal fraction with mitochondria and/or peroxisomes was also considered as a potential source of error in the estimation of Δ6-desaturase. The activity of succinate dehydrogenase, a marker of mitochondrial contamination, was low and did not differ significantly between control and cholesterol fed rats. In addition, the products generated by peroxisomal β-oxidation (mainly acetate and water soluble compounds), would not interfere with the estimation of Δ6-desaturase as they are not extracted into the solvent phase.

Packed column GLC was used to separate tissue fatty acid methyl esters for subsequent calculation of Δ6-desaturase indices. This technique does not separate 18:3 n-6 from 20:0 and so strictly speaking the 18:3 n-6/18:2 n-6 Δ6-desaturase ratio is incorrect. Nevertheless most of this peak contains 18:3 n-6 (approximately 80 % estimated by capillary GLC) and in the first cholesterol feeding study (Chapter 4) there was no systematic effect on the amount of 20:0 in microsomal phospholipids suggesting that this is not a major source of error.

Therefore by establishing a very sensitive technique to measure microsomal Δ6-desaturase activity and by limiting the extent to which substrate oxidation affects its measurement, it was possible to assess accurately the effects of specific factors on linoleic acid Δ6-desaturation in the rat. Comparisons between Δ6-desaturase activity and tissue fatty acid compositions (product-precursor ratios) are discussed below.

Sprague Dawley rats subjected to isolation stress had a lower liver microsomal Δ6-desaturase activity than controls and this finding was recently confirmed in normotensive (Wistar Kyoto) and spontaneously hypertensive rats by Mills *et al.* (1994). Diabetes, generally assumed to be associated with a reduced Δ6-desaturase activity, was also investigated. Diabetic models using chemical diabetogenic agents were avoided as it is questionable whether these models adequately reflect the situation presented in humans (Marliss *et al.*, 1982; Baird, 1989). Animals that spontaneously

develop autoimmune diabetes, such as the BB/E diabetic rat, have distinct advantages over these earlier models in that the disease exhibited closely resembles that found in Type I human diabetics (Baird, 1989). Moreover, insulin can be administered at varying levels to achieve different degrees of diabetic control. Under conditions of regular insulin treatment BB/E diabetic rats had a 25 % lower liver microsomal Δ6-desaturase activity compared to non-diabetic controls. The activity of Δ6-desaturase was reduced slightly further in BB/E diabetic rats in whom insulin treatment was withdrawn for 54 h. An important finding in the insulin withdrawn group was the marked elevation of microsomal non-esterified 18:2 n-6 levels which possibly explains previous inconsistent published results. By not using the correct specific activity one would in fact underestimate the activity of Δ6-desaturase in diabetic animals. This could therefore cast doubt on the correctness of some of the published results. The concentration of microsomal non-esterified 18:2 n-6 in the present optimised assay system, although theoretically important, did not seriously affect the activity of Δ6-desaturase. Clearly, assay conditions are important for an accurate assessment of the activity of this enzyme.

Other work on risk factors, examining the role of dietary cholesterol supplementation on Δ6-desaturase activity, consistently demonstrated, though contrary to expectations, a significant *increase* in activity compared with controls. This result was observed when the optimised assay for Δ6-desaturase (developed for this project) was used as well as when the sub-optimal assay conditions as detailed by Garg *et al.* (1988a) were deliberately employed. Sub-optimal measurements exhibited 30-fold lower Δ6-desaturase activities and were characterised by a larger standard deviation of replicate analyses. Differences in the level of microsomal non-esterified 18:2 n-6 between optimal and sub-optimal assay conditions could not explain these divergent results. Furthermore, isolation of rats believed to alter the effect of cholesterol feeding on Δ6-desaturase did not modify the cholesterol-induced increase in activity.

Measurements of Δ6-desaturase *in vitro* and *in vivo* gave constrasting results. Data from the *in vitro* assay demonstrated a cholesterol-induced increase in activity. The

results obtained *in vivo* indicated the opposite. A number of reasons may explain this difference. For example the *in vitro* assay was designed to obtain maximum Δ6-desaturase activity by providing saturating substrate and cofactors; a situation not present *in vivo*. In addition, certain inhibitors of the Δ6-desaturative process *in vivo* may be too dilute to interfere with the process *in vitro* or may have been lost during microsomal preparation. Until this has been fully resolved, *in vitro* data on its own should be considered with some caution.

Product-precursor ratios of fatty acids esterified to microsomal phospholipids, frequently used to assess the activity of a particular fatty acid metabolic conversion, were also examined in the above studies. For example, the 18:3 n-6/18:2 n-6 ratio (Δ6-desaturase index) in BB/E diabetic rats, treated regularly with insulin, was increased compared with controls (direct Δ6-desaturase measurements showed a 25 % *decrease* in activity). However, the same ratio did not differ from controls in diabetic animals in whom insulin was withdrawn, although Δ6-desaturase activity was 39 % lower in these rats compared with controls. In contrast, the 22:5 n-6/22:4 n-6 ratio, which also reflects Δ6-desaturase activity, was decreased in insulin treated diabetic rats and reduced slightly further by insulin withdrawal. Indeed, this fatty acid ratio correlated well with microsomal Δ6-desaturase activity as well as with an overall index of 18:2 n-6 metabolism (calculated from adding all microsomal phospholipid n-6 fatty acids derived from 18:2 n-6 and dividing by 18:2 n-6). It is therefore evident that n-6 fatty acid compositions and the derived fatty acid ratios, do not adequately relate to the activity of Δ6-desaturase in liver microsomes.

Adipose triacylglycerol and plasma phospholipid fatty acid compositions, used in human studies as indicators of intake and metabolism of 18:2 n-6 (Riemersma *et al.*, 1986; Wood *et al.*, 1987), showed small, inconsistent changes in our rats. Isolation did not affect these compositions at all. Such variable results with two CHD risk factors occurred despite the fact that these studies were tightly controlled using inbred strains and semi-synthetic diets. It is therefore unlikely that in human studies, where variations in both dietary intake and genetic origin occur, the difference observed with Δ6-

desaturase indices based on plasma and adipose tissue fatty acid composition can be interpreted to indicate the activity of this pathway. Of course, adipose 18:2 n-6 is generally accepted as a good indicator of long term dietary intake of this EFA in an individual but the situation is further complicated by specific dietary advice given to sections of the community (diabetics, hyperlipidaemics, and other subjects with a high risk of CHD). In the absence of a valid marker (i.e. Δ6-desaturase index on the basis of fatty acid compositions of plasma or adipose tissue) that can be used in population studies, extrapolation to the human situation is virtually impossible for Δ6-desaturase.

Extrapolation of rat data to humans, cannot be necessarily justified also in view of major differences between species in Δ6-desaturase activity, in resistance to cholesterol feeding and in other aspects of lipoprotein metabolism. Future studies should therefore be carried out to assess the effect of CHD risk factors on Δ6-desaturase activity in humans. Obviously, the use of liver biopsies from human volunteers for Δ6-desaturase estimation is not ethical and alternative methods are required. Appraisal of 18:2 n-6 metabolism *in vivo* using [^{13}C]18:2 n-6 (Emken *et al.*, 1987; Emken *et al.*, 1990) is not widely used because of the high cost of the stable isotope and associated separation and detection equipment (gas liquid chromatography-mass spectrometry). In addition the method does not give a quantitative measure of whole body Δ6-desaturase activity. Another method that is potentially applicable to human and epidemiological studies is the measurement of Δ6-desaturase in circulating blood cells. Cunnane has claimed that isolated white blood cells have measurable activity (Cunnane *et al.*, 1984). However, this methodology has not been validated in humans using stable isotopes or in animals by measuring both liver microsomal and white blood cell Δ6-desaturase activity. However, if suitable cells exist in which Δ6-desaturase activity reflected that of the liver then raising antibodies to the terminal Δ6-desaturase protein of such cells may provide another potential way for the assessment of an individual's ability to metabolise 18:2 n-6. This latter method, which necessitates isolation and purification of the human Δ6-desaturase enzyme complex, could be very useful and permit

differentiation between active and inactive forms of the enzyme and so allow the identification of factors that induce enzyme synthesis and activation.

Extrapolation of data derived from the rat cholesterol feeding experiments to humans may be difficult to justify. The typical cholesterol content in the human diet is in the order of 300 mg per day, equivalent to approximately 4 mg/kg body weight/day. The cholesterol enriched diet given to rats in the present study was equivalent to approximately 200 mg/kg body weight/day. It is therefore evident that these animals consumed much greater amounts of cholesterol than that ever naturally consumed by humans. However, plasma cholesterol levels increased only modestly, suggesting that the use of this type of method can be justified (A similar dietary regimen causes plasma cholesterol levels in excess of 45 mM in rabbits; Weidinger *et al.*, 1991). The use of dietary cholesterol in the present study, however, has mainly been as a *tool* to assess the effect of a specific dietary factor on Δ6-desaturation of 18:2 n-6.

The project described in this thesis did not examine the relation between impaired 18:2 n-6 Δ6-desaturation and CHD, directly. In view of the fact that it is still uncertain whether the activity in the liver microsomes *in vitro* reflects that *in vivo*, as discussed above, it would be hasty to draw wide ranging conclusions. Nevertheless it is worthwhile to reconsider this hypothesis in some detail.

First of all, the results suggest that dietary cholesterol possibly decreases the activity of Δ5-desaturase more than that of Δ6-desaturase. This was observed in the *in vivo* experiments in particular. Furthermore, the overall index of 18:2 n-6 desaturation and elongation was consistently decreased despite increased or decreased Δ6-desaturase activity in the cholesterol fed or diabetic animals. This suggests that other factors that control the metabolism of 18:3 n-6, whether elongation followed by desaturation by Δ5-desaturase or incorporation into phospholipids are affected to a larger degree than the so called rate limiting step of Δ6-desaturase. This raises the question whether Δ6-desaturase should be linked with the development of CHD in the first place.

There are several mechanisms whereby a low activity of this enzyme could increase the risk of CHD. γ-Linolenic acid has been claimed to lower serum cholesterol levels (Horrobin and Manku, 1983; Horrobin, 1992). However, human studies using a range of 18:3 n-6 intakes (by supplementing with evening primrose oil) do not support this finding (Abraham *et al.*, 1990). It is also unlikely that the activity of Δ6-desaturase could influence tissue levels of 20:4 n-6 and hence the production of prostanoids in a major way. First of all careful dietary feeding studies using 18:2 n-6 rich diets have shown that it is virtually impossible to change the amount of 20:4 n-6 in cardiac phospholipids (with the exception of fish oil; Sargent, 1990). The effect of prostacyclin and thromboxane A_2, both formed from phospholipid 20:4 n-6, differ in their effects on the cardiovascular system. Prostacyclin inhibits platelet aggregation, induces vasodilation and prevents ischaemic arrhythmias, whilst thromboxane A_2 does essentially the opposite. Therefore it is the balance between prostacyclin and thromboxane A_2 that determines whether there is a pro-thrombotic of pro-arrhythmic effect. Secondly, the amount of 20:4 n-6 released is under the control of phospholipase A_2 and the levels of prostacyclin and thromboxane A_2 produced are controlled by prostanoid synthase. Thus it is questionable that the relative amount of 20:4 n-6 could be the main determinant of prostanoid production. Recent studies suggest there are differences in constitutive and inducible forms of prostanoid synthase (PGHS-1 and PGHS-2) and if the analogy with nitric oxide is valid (Xie and Nathan, 1994) it may be that the factors that cause an induction of PGHS-2 are more closely related to the pathological changes and inflammation than the activity of PGHS-1 itself (Hla, 1992; Vane, 1994).

Perhaps there are other explanations for the apparent association between low Δ6-desaturase and CHD. Low Δ6-desaturase activity is proposed to be associated with diets low in EFA and rich in SFA. Under these conditions, the inverse association between adipose 16:1 and 18:2 n-6 has been observed for a long time (Kingsbury *et al.*, 1974; Wood *et al.*, 1984). Furthermore, if an increased Δ9-desaturation of 18:0 occurs the resulting elevated content of 18:1 could overwhelm the Δ6-desaturation of

18:2 n-6. Similarly, lack of access of 18:2 n-6 to the enzyme, perhaps because it is conserved within the microsomal phospholipid pool, may decrease 20:4 n-6 levels. As a result 20:3 n-9 levels are increased. This end product cannot be converted to 20:4 n-9 as humans and other mammals cannot insert a double bond between C_2 and C_3 as they do not possess microsomal Δ2-desaturase. Elevated levels of 20:3 n-9 are present in platelets of individuals that have sustained an acute myocardial infarction (Wood *et al.*, 1987) and this fatty acid is linked with platelet hyperactivity (Nordøy *et al.*, 1985). Indeed, increased levels of the pro-thrombotic 12-lipoxygenated derivative of 20:3 n-9 are formed under thrombin stimulated platelet aggregation (Lagarde *et al.*, 1985).

Human diets rich in SFA also tend to be rich in animal fats and cholesterol. Dietary cholesterol has little influence, however, on plasma total cholesterol levels (Bronsgeest-Schoute *et al.*, 1979a; Bronsgeest-Schoute *et al.*, 1979b; McGill, 1979), although it is now recognised that the individual response to dietary cholesterol differs considerably and is under genetic control (apo-lipoprotein E polymorphisms). In susceptible individuals if raised cholesterol does inhibit Δ6-desaturase, then this could reinforce the effect of SFA on Δ9-desaturation and stimulate the formation of 20:3 n-9 further. It is noteworthy, where both dietary cholesterol and SFA are combined with fish oil, the inhibitory effect of fish oil on Δ9- and Δ6-desaturase activities prevents a rise in tissue 20:3 n-9 levels (Garg *et al.*, 1988a).

Much of this is very speculative. Direct measurements of Δ6-desaturase activity in CHD case control studies, in which classical risk factors will also be measured, are needed. This to examine if low Δ6-desaturase is indeed a new, independent risk factor for CHD. As the nature of such epidemiological evidence is still circumstantial, additional proof would be required. The role of Δ6-desaturase in predicting development of CHD in prospective and intervention studies, aimed at reducing factors that inhibit Δ6-desaturase in humans, need to be examined. Until then, no firm claim can be made that low Δ6-desaturase activity causes increased CHD mortality.

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Appendices

A-G

Appendix A

Ingredients for the dietary salt mix (2.677 kg) :-

Compound	**Supplier**	**Amount (g)**
Calcium carbonate ($CaCO_3$)	BDH	1007
Copper chloride ($CuCl_2.2H_2O$)	Sigma	2.35
Ferric citrate ($FeC_6H_5O_7$)	Sigma	21.95
Magnesium phosphate ($MgHPO_4.3H_2O$)	Sigma	478
Manganese sulphate ($MnSO_4$)	Sigma	33.9
Trisodium citrate ($C_6H_5O_7Na_3.3H_2O$)	Sigma	355.5
Potassium hydrogen carbonate ($KHCO_3$)	BDH	359.5
Potassium chloride (KCl)	BDH	175
Potassium dihydrogen phosphate (KH_2PO_4)	Sigma	237.5
Potassium iodate (KIO_3)	BDH	0.035
Zinc chloride ($ZnCl_2$)	Sigma	6.25

Appendix B

Ingredients for the dietary vitamin mix (1.5 kg) :-

Compound	**Supplier**	**Amount (g)**
Calcium pantothenic acid	Sigma	7.5
Choline chloride	Sigma	750
Folic acid	BDH	0.375
Menadione	BDH	0.375
Myo-inositol	Sigma	37.5
Nicotinic acid	Sigma	7.5
Vitamin A (Retinol acetate)	Sigma	0.588
Vitamin B_1 (Thiamine hydrochloride)	Sigma	2.250
Vitamin B_2 (Riboflavin)	Sigma	2.250
Vitamin B_6 (Pyridoxine monohydrochloride)	Sigma	0.75
Vitamin B_{12} (Cyanocobalamin)	BDH	0.008
Vitamin D_2 (Calciferol)	BDH	0.140
Vitamin H (d-Biotin)	Sigma	0.075

Made up to 1.5 kg with fumed silica (75 g) and sucrose (615.7 g).

Appendix C

Ingredients for the diets (2.0 kg) :-

Compound	Supplier	Amount (g)
Cornflour	A.Clark Food Service, Glasgow, U.K.	478
Casein	Scottish Pride Quality Dairy Foods, Paisley, U.K.	540
Glucose	BDH	414
Cellulose	BDH	100
Beef tallow	A.Clark Food Service, Glasgow, U.K.	360
Safflower oil	Lipid Teknik, Stockholm, Sweden.	40
Mineral mix	see Appendix A	46.6
Vitamin mix	see Appendix B	8.6
(+)-α-tocopherol	Sigma	0.106
L-methionine	Sigma	12.5

Appendix D

Cobas Bio Centrifugal analyser details:-

No.	Command	Protein assay	ATP assay	CP assay
1	Units	μg/ml	μmol/l	μmol/l
2	Calculation factor	1494	0	0
3	Standards	100, 200, 300, 400, 500	684, 684, 684	1125, 1125, 1125
6	Limit	0	2000	2000
7	Temperature. (°C)	37	37	37
8	Analysis type	7.6	6	6
9	Wavelength (nm)	750	340	340
10	Sample volume (μl)	10	15	0
11	Diluent volume (μl)	30	15	0
12	Reagent volume (μl)	240	340	0
13	Incubation time (s)	600	80	10
14	Start reagent volume (μl)	45	8	8
15	Time of first reading (s)	0.5	60	60
16	Time interval (s)	60	10	10
17	Number of readings	7	10	10
18	Blanking mode	1	1	5
19	Printout mode	5	1	1

No.	Command	Cholesterol assay	Triacylglycerol assay
1	Units	mmol/l	mmol/l
2	Calculation factor	0	0
3	Standards	8.82, 8.82, 8.82	3.58, 3.58, 3.58
6	Limit	10	7
7	Temperature. (°C)	37	37
8	Analysis type	1	6
9	Wavelength (nm)	500	340
10	Sample volume (μl)	5	8
11	Diluent volume (μl)	40	40
12	Reagent volume (μl)	340	320
13	Incubation time (s)	0	120
14	Start reagent volume. (μl)	0	8
15	Time of first reading (s)	0.5	0.5
16	Time interval (s)	20	600
17	Number of readings	19	02
18	Blanking mode	1	1
19	Printout mode	1	1

Appendix E

Table E.1 *Body and liver weights of rats fed a control or cholesterol enriched diet for 30 days at 8, 24 and 48 h after administration of radioactive fatty acids*

Measurement	Time (h)	Control	Chol.	Significance
Weight (g)				
Body	8	268±14	259±27	n.s.
	24	275±23	266±55	n.s.
	48	291±44	282±43	n.s.
Liver	8	8.7±0.8	10.4±1.3	*
	24	11.6±1.2	12.6±2.7	n.s.
	48	11.4±1.8	12.3±2.3	n.s.
Relative weight (%)	8	3.2±0.1	4.0±0.1	***
	24	4.2±0.2	4.7±0.4	*
	48	3.9±0.1	4.4±0.3	*

Values expressed as Mean±SD. * $p<0.05$, *** $p<0.001$ *vs.* control.

Table E.2 *Microsomal cholesterol and phospholipid contents and Δ6-desaturase activity of rats fed a control or cholesterol enriched diet for 30 days at 8, 24 and 48 h after administration of radioactive fatty acids*

Measurement	Time (h)	Control	Chol.	Significance
Cholesterol ξ	8	$88±7^{a}$	$108±19^{a}$	n.s.
	24	$75±8^{b}$	$114±28^{a}$	*
	48	$69±8^{b}$	$92±15^{a}$	*
Phospholipid ξ	8	$463±23^{a}$	$462±30^{a}$	n.s.
	24	$456±26^{a}$	$487±36^{a,b}$	n.s.
	48	$497±20^{b}$	$502±20^{b}$	n.s.
Cholesterol/phospholipid ψ	8	$0.19±0.02^{a}$	$0.23±0.05^{a}$	n.s.
	24	$0.17±0.02^{a,b}$	$0.23±0.05^{a}$	*
	48	$0.14±0.02^{b}$	$0.18±0.04^{a}$	*
Δ6-desaturase activity ζ	8	$490±82^{a}$	$611±128^{a}$	n.s.
	24	$466±43^{a}$	$455±105^{b}$	n.s.
	48	$455±45^{a}$	$470±69^{b}$	n.s.

Values expressed as Mean±SD. * $p<0.05$ *vs.* control. ξ nmol/mg protein, ψ mol/mol, ζ Δ6-desaturase activity estimated with 0.5 mg microsomal protein (activity expressed as pmol/min/mg). Single a, b, c unlike symbols indicate statistical significance ($p<0.05$) between two groups in the vertical plane for a particular measurement by unpaired t-test after significant ($p<0.05$) one-way ANOVA.

Appendix E

Table E.3 *Microsomal total phospholipid fatty acid composition of rats fed a control or cholesterol enriched diet for 30 days at 8, 24 and 48 h after administration of radioactive fatty acids*

Fatty acid species	Time (h)	Control	Chol.	Significance
18:0	8	265±15	265±15	n.s.
	24	215±17	220±40	n.s.
	48	263±15	237±31	n.s.
18:1	8	89±8	107±8	**
	24	109±12	144±19	**
	48	103±10	142±6	***
20:1	8	1.2±0.1	1.4±0.2	*
	24	1.6±0.5	3.0±1.0	*
	48	2.0±0.7	3.1±0.7	*
n-6				
18:2	8	79±11	106±11	**
	24	109±9	146±19	**
	48	106±6	138±9	***
18:3 #	8	0.6±0.3	0.9±0.3	n.s.
	24	2.3±0.7	2.0±0.4	n.s.
	48	1.3±0.2	1.5±0.3	n.s.
20:3	8	8.0±2.0	14±3	**
	24	16±1	22±3	**
	48	19±2	26±1	***
20:4	8	240±9	206±19	**
	24	192±13	170±13	*
	48	226±11	193±7	***
22:4	8	2.7±0.2	1.9±0.3	***
	24	2.4±0.5	1.8±0.8	**
	48	2.6±0.5	1.8±0.2	**
22:5	8	6.4±1.8	3.5±1.7	*
	24	5.9±1.6	4.3±1.5	n.s.
	48	11±3	5.4±1.2	*

Values expressed as nmol/mg microsomal protein (Mean±SD). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs.* control. # Unresolved peak contained unspecified amounts of 20:0.

Appendix E

Table E.4 *Microsomal total phospholipid fatty acid composition of rats fed a control or cholesterol enriched diet for 56 days at 8, 24 and 48 h after administration of radioactive fatty acids*

Fatty acid species	Time (h)	Control	Chol.	Significance
18:0	8	259±24^{a}	277±18^{a}	n.s.
	24	218±18^{b}	196±14^{b}	*
	48	232±19^{b}	205±15^{b}	*
18:1	8	83±6^{a}	115±9^{a}	***
	24	102±7^{b}	138±8^{b}	***
	48	100±7^{b}	150±11^{b}	***
20:1	8	1.1±0.2^{a}	1.6±0.3^{a}	*
	24	1.4±0.2^{b}	3.0±0.5^{b}	***
	48	1.9±0.2^{c}	3.0±0.6^{b}	**
n-6				
18:2	8	86±11^{a}	117±7^{a}	***
	24	99±5^{b}	129±9^{b}	***
	48	94±7^{a,b}	120±7^{a,b}	***
18:3 #	8	0.9±0.1^{a}	1.1±0.1^{a}	**
	24	3.2±0.6^{b}	2.7±0.3^{b}	n.s.
	48	1.9±0.2^{c}	1.6±0.4^{c}	n.s.
20:3	8	8.6±1.2^{a}	15±1^{a}	***
	24	16±1^{b}	20±1^{b}	***
	48	18±2^{c}	22±3^{b}	*
20:4	8	231±29^{a}	223±28^{a}	n.s.
	24	199±14^{b}	175±10^{b}	**
	48	205±16^{a,b}	188±15^{b}	n.s.
22:4	8	2.5±0.3	1.9±0.3	*
	24	2.8±0.3	1.8±0.2	***
	48	2.4±0.3	1.7±0.2	**
22:5	8	4.4±0.8^{a}	3.4±2.3	n.s.
	24	7.0±2.1^{b}	3.6±1.1	***
	48	8.4±2.0^{b}	4.7±1.3	**

Values expressed as nmol/mg microsomal protein (Mean±SD). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs.* control. # Unresolved peak contained unspecified amounts of 20:0. Single a, b, c unlike symbols indicate statistical significance ($p<0.05$) between two groups in the vertical plane for a particular measurement by unpaired t-test after significant ($p<0.05$) one-way ANOVA.

Appendix E

Table E.5 *Microsomal phospholipid fatty acid ratios of rats fed a control or cholesterol enriched diet for 30 days at 8, 24 and 48 h after administration of radioactive fatty acids*

Fatty acid ratio	Time (h)	Control	Chol.	Significance
n-6				
18:3/18:2 (Δ6)	8	0.008 ± 0.003^{a}	0.009 ± 0.002^{a}	n.s.
	24	0.021 ± 0.006^{b}	0.014 ± 0.004^{b}	n.s.
	48	0.012 ± 0.002^{c}	$0.011\pm0.003^{a,b}$	n.s.
20:4/20:3 (Δ5)	8	31.6 ± 7.6^{a}	15.1 ± 2.8^{a}	**
	24	12.3 ± 0.8^{b}	7.8 ± 1.4^{b}	***
	48	11.7 ± 0.8^{b}	7.4 ± 0.4^{b}	***
22:5/22:4 (Δ6)	8	2.4 ± 0.5^{a}	1.7 ± 0.6^{a}	n.s.
	24	2.4 ± 0.6^{a}	$2.4\pm0.6^{a,b}$	n.s.
	48	4.0 ± 0.8^{b}	3.1 ± 0.6^{b}	*
18:2 D&E	8	3.3 ± 0.3^{a}	2.1 ± 0.2^{a}	***
	24	2.0 ± 0.2^{b}	1.4 ± 0.2^{b}	***
	48	2.5 ± 0.3^{c}	1.7 ± 0.1^{c}	***
n-9				
18:1/18:0 (Δ9)	8	0.33 ± 0.03^{a}	0.40 ± 0.03^{a}	**
	24	0.51 ± 0.08^{b}	0.67 ± 0.16^{b}	n.s.
	48	0.39 ± 0.03^{c}	0.61 ± 0.10^{b}	**

Values expressed as Mean±SD. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs.* control. Single a, b, c unlike symbols indicate statistical significance ($p<0.05$) between two groups in the vertical plane for a particular measurement by unpaired t-test after significant ($p<0.05$) one-way ANOVA. 18:2 D (Desaturation) & E (Elongation) refers to (18:3 n-6 + 20:2 n-6 + 20:3 n-6 + 20:4 n-6 + 22:4 n-6 + 22:5 n-6)/18:2 n-6.

Appendix E

Table E.6 *Distribution of ^{14}C in microsomal phospholipid fatty acids of rats fed a control or cholesterol enriched diet for 30 days at 8, 24 and 48 h after administration of radioactive fatty acids*

Label	Time (h)	Control	Chol.	Significance
[^{14}C]phospholipid	8	3027±843	3330±1320	n.s.
	24	1341±518	1579±447	n.s.
	48	714±138	741±132	n.s.
[^{14}C]dienes	8	2616±771	2901±1151	n.s.
	24	877±384	1183±343	n.s.
	48	242±46	337±48	**
[^{14}C]trienes	8	181±66	222±103	n.s.
	24	180±75	207±70	n.s.
	48	91±15	129±24	**
[^{14}C]tetraenes	8	171±34	103±38	*
	24	254±88	123±34	*
	48	363±78	243±73	*
[^{14}C]SFA & [^{14}C]MUFA	8	59±28	105±37	*
	24	31±6	66±35	n.s.
	48	18±5	32±11	*

Values expressed as DPM/mg microsomal protein (Mean±SD). * $p<0.05$, ** $p<0.01$ *vs.* control.

Table E.7 *Specific activities of microsomal phospholipid [^{14}C]labelled n-6 fatty acids of rats fed a control or cholesterol enriched diet for 30 days at 8, 24 and 48 h after administration of [1-^{14}C]18:2 n-6*

Measurement	Time (h)	Control	Chol.	Significance
N-6 diene specific activity	8	32.5±6.5	26.9±9.1	n.s.
	24	7.9±3.0	8.1±2.0	n.s.
	48	2.3±0.4	2.4±0.3	n.s.
N-6 triene specific activity	8	20.7±2.9	14.5±4.7	*
	24	9.9±3.7	8.4±1.8	n.s.
	48	4.4±0.9	4.7±0.7	n.s.
N-6 tetraene specific activity	8	0.7±0.1	0.5±0.2	n.s.
	24	1.3±0.4	0.7±0.2	*
	48	1.6±0.3	1.2±0.3	n.s.

Values expressed as DPM/nmol (Mean±SD). For dienes the denominator is 18:2 n-6 plus 20:2 n-6 (<1.0 nmol 20:2 n-6 per mg microsomal protein), for trienes the denominator is 18:3 n-6 plus 20:3 n-6 and for tetraenes the denominator is 20:4 n-6 plus 22:4 n-6. * $p<0.05$ *vs.* control.

Appendix E

Table E.8 *Distribution of ^3H in microsomal phospholipid fatty acids of rats fed a control or cholesterol enriched diet for 30 days at 8, 24 and 48 h after administration of [11,12-^3H]18:0*

Measurement	Time (h)	Control	Chol.	Significance
[^{3}H]phospholipid	8	3280±417	2653±521	*
	24	1310±388	1382±393	n.s.
	48	481±169	496±151	n.s.
[^{3}H]SFA	8	3280±417	2638±519	*
	24	1256±372	1282±391	n.s.
	48	459±149	449±141	n.s.
[^{3}H]MUFA	8	n.d.	15±14	-
	24	55±36	100±48	n.s.
	48	21±11	47±22	*

Values expressed as DPM/mg microsomal protein (Mean±SD). * $p<0.05$ *vs.* control. n.d. not detectable. - Not statistically feasible.

Table E.9 *Specific activity of microsomal phospholipid [^3H]labelled fatty acids of rats fed a control or cholesterol enriched diet for 30 days at 8, 24 and 48 h after administration of [11,12-^3H]18:0*

Measurement	Time (h)	Control	Chol.	Significance
SFA specific activity	8	7.6±0.7	6.2±1.2	*
	24	3.1±0.8	3.1±0.6	n.s.
	48	1.0±0.3	1.1±0.3	n.s.
MUFA specific activity	8	n.d.	0.1±0.1	-
	24	0.4±0.2	0.6±0.2	n.s.
	48	0.2±0.1	0.3±0.1	n.s.

Values expressed as DPM/nmol (Mean±SD). * $p<0.05$. For SFA specific activity the denominator is total microsomal SFA, for MUFA specific activity the denominator is total microsomal MUFA. - Not statistically feasible.

Appendix E

Table E.10 *Plasma total cholesterol, cholesterol ester, triacylglycerol and phospholipid concentrations of rats fed a control or cholesterol enriched diet for 30 days at 8, 24 and 48 h after administration of radioactive fatty acids*

Measurement	Time (h)	Control	Chol.	Significance
Total cholesterol	8	1.7±0.2^{a}	2.1±0.3^{a}	n.s.
	24	1.8±0.5^{a}	2.5±0.4^{a}	*
	48	2.1±0.3^{a}	3.3±0.5^{b}	***
Cholesterol ester	8	1.4±0.2^{a}	1.7±0.2^{a}	*
	24	1.4±0.3^{a}	1.9±0.3^{a}	*
	48	1.6±0.2^{a}	2.5±0.3^{b}	***
Triacylglycerol	8	1.2±0.4^{a}	1.3±0.2^{a}	n.s.
	24	2.0±1.0^{a}	1.8±0.4^{a}	n.s.
	48	1.8±0.6^{a}	1.7±0.6^{a}	n.s.
Phospholipid	8	1.4±0.2^{a}	1.4±0.2^{a}	n.s.
	24	1.5±0.2^{a}	1.6±0.1^{a,b}	n.s.
	48	1.6±0.2^{a}	1.6±0.1^{b}	n.s.

Values expressed as mmol/l (Mean±SD). * $p<0.05$, *** $p<0.001$ *vs.* control. a, b, c unlike symbols indicate statistical significance ($p<0.05$) between two groups in the vertical plane for a particular measurement by unpaired t-test after significant ($p<0.05$) one-way ANOVA.

Table E.11 *Plasma total cholesterol, cholesterol ester, triacylglycerol and phospholipid concentrations of rats fed a control or cholesterol enriched diet for 56 days at 8, 24 and 48 h after administration of radioactive fatty acids*

Measurement	Time (h)	Control	Chol.	Significance
Total cholesterol	8	1.6±0.2	1.7±0.3^{a}	n.s.
	24	1.8±0.4	2.3±0.5^{b}	n.s.
	48	2.0±0.3	3.6±0.9^{c}	**
Cholesterol ester	8	1.3±0.3	1.5±0.2^{a}	n.s.
	24	1.6±0.3	2.0±0.4^{b}	n.s.
	48	1.7±0.3	2.7±0.5^{c}	**
Triacylglycerol	8	1.5±0.7	1.2±0.5^{a}	n.s.
	24	1.8±0.7	2.1±0.5^{b}	n.s.
	48	1.7±0.5	1.4±0.4^{a}	n.s.
Phospholipid	8	1.3±0.2	1.1±0.1^{a}	n.s.
	24	1.4±0.2	1.5±0.3^{b}	n.s.
	48	1.5±0.2	1.5±0.2^{b}	n.s.

Values expressed as mmol/l (Mean±SD). ** $p<0.01$ *vs.* control. Single a, b, c unlike symbols indicate statistical significance ($p<0.05$) between two groups in the vertical plane for a particular measurement by unpaired t-test after significant ($p<0.05$) one-way ANOVA.

Appendix E

Table E.12 *Plasma phospholipid fatty acid composition of rats fed a control or cholesterol enriched diet for 30 days* §

Fatty acid species	Control	Chol.	Significance
SFA			
14:0	6.7±1.4	9.6±2.3	*
16:0	659±76	644±47	n.s.
18:0	737±122	670±62	n.s.
MUFA			
16:1	31±6	52±13	*
18:1	400±58	524±47	**
20:1	6.8±1.6	11±3	*
PUFA (n-6)			
18:2	679±90	753±74	n.s.
20:2	3.6±1.2	4.4±0.6	n.s.
18:3	4.2±1.1	5.0±0.9	n.s.
20:3	64±12	83±5	*
20:4	452±105	388±42	n.s.
22:4	6.0±3.1	4.9±0.9	n.s.
22:5	23±9	13±4	*
PUFA (n-3)			
18:3	1.0±0.5	0.8±0.4	n.s.
20:5	5.2±2.3	6.4±2.4	n.s.
22:5	7.9±2.8	7.3±1.9	n.s.
22:6	84±28	68±5	n.s.
PUFA (n-9)			
20:3	35±11	29±4	n.s.
Σ SFA	1403±194	1324±75	n.s.
Σ MUFA	438±64	587±61	**
Σ n-6	1231±192	1252±83	n.s.
Σ n-3	98±33	82±8	n.s.

Values expressed as μmol/l (Mean±SD). * $p<0.05$, ** $p<0.01$ *vs.* control. § All measurements made 48 h after administration of radioactive fatty acids.

Appendix E

Table E.13 *Plasma phospholipid fatty acid composition of rats fed a control or cholesterol enriched diet for 56 days* §

Fatty acid species	Control	Chol.	Significance
SFA			
14:0	7.9±1.6	8.8±1.5	n.s.
16:0	648±113	595±99	n.s.
18:0	690±98	569±67	*
MUFA			
16:1	35±5	55±11	**
18:1	412±81	521±91	n.s.
20:1	6.5±1.6	9.1±1.5	*
PUFA (n-6)			
18:2	594±93	625±104	n.s.
20:2	2.9±0.9	3.5±1.1	n.s.
18:3 #	5.1±1.3	5.0±1.0	n.s.
20:3	65±10	69±10	n.s.
20:4	451±65	377±28	*
22:4	7.3±0.9	5.0±0.6	***
22:5	21±6	11±3	*
PUFA (n-3)			
18:3	0.8±0.5	0.6±0.3	n.s.
20:5	6.4±1.4	7.8±2.3	n.s.
22:5	9.3±1.1	6.8±1.2	**
22:6	76±13	55±10	*
PUFA (n-9)			
20:3	42±14	34±6	n.s.
Σ SFA	1346±204	1172±161	n.s.
Σ MUFA	453±87	585±102	*
Σ n-6	1146±166	1096±133	n.s.
Σ n-3	93±14	70±11	*

Values expressed as μmol/l (Mean±SD). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs.* control. # Unresolved peak contained unspecified amounts of 20:0. § All measurements made 48 h after administration of radioactive fatty acids.

Appendix E

Table E.14 *Plasma cholesterol ester fatty acid composition of rats fed a control or cholesterol enriched diet for 30 days* §

Fatty acid species	Control	Chol.	Significance
SFA			
16:0	149±19	283±46	***
18:0	16±3	63±8	***
MUFA			
16:1	52±12	195±69	**
18:1	214±36	987±198	***
20:1	2.6±0.9	21±8	**
PUFA (n-6)			
18:2	427±66	422±88	n.s.
18:3	15±4	15±4	n.s.
20:3	17±5	19±10	n.s.
20:4	700±108	438±122	**
22:5	1.5±1.0	0.8±0.5	n.s.
PUFA (n-3)			
18:3	0.8±0.3	2.0±0.5	**
20:5	17±7	19±10	n.s.
22:6	16±3	10±3	**
PUFA (n-9)			
20:3	19±6	11±2	*
Σ SFA	165±21	347±51	***
Σ MUFA	268±46	1203±272	***
Σ n-6	1160±159	894±208	*
Σ n-3	34±9	31±12	n.s.

Values expressed as μmol/l (Mean±SD). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs.* control. § All measurements made 48 h after administration of radioactive fatty acids.

Appendix E

Table E.15 *Plasma cholesterol ester fatty acid composition of rats fed a control or cholesterol enriched diet for 56 days* §

Fatty acid species	Control	Chol.	Significance
SFA			
16:0	131±18	301±73	**
18:0	12±2	68±22	**
MUFA			
16:1	59±7	229±65	**
18:1	198±30	1171±385	**
20:1	4.6±2.5	28±6	***
PUFA (n-6)			
18:2	399±53	381±54	n.s.
18:3 #	19±2	15±4	n.s.
20:3	22±6	20±3	n.s.
20:4	753±144	448±60	**
22:4	0.9±1.0	0.8±1.0	n.s.
22:5	2.6±1.4	1.2±0.8	n.s.
PUFA (n-3)			
18:3	0.4±0.4	2.5±0.9	**
20:5	26±3	25±5	n.s.
22:6	19±5	11±3	*
PUFA (n-9)			
20:3	25±9	15±3	n.s
Σ SFA	143±20	368±95	**
Σ MUFA	262±37	1428±452	**
Σ n-6	1196±199	865±106	**
Σ n-3	45±6	38±6	n.s.

Values expressed as μmol/l (Mean±SD). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs.* control. # Unresolved peak contained unspecified amounts of 20:0. § All measurements made 48 h after administration of radioactive fatty acids.

Appendix E

Table E.16 *Adipose tissue triacylglycerol fatty acid composition of rats fed a control or cholesterol enriched diet for 30 days* §

Fatty acid species	Control	Chol.	Significance
SFA			
14:0	2.3±0.2	2.3±0.2	n.s.
16:0	23.1±0.6	22.5±0.6	n.s.
18:0	11.2±0.9	11.0±1.0	n.s.
20:0	0.1±0.0	0.1±0.0	n.s.
MUFA			
16:1	4.2±0.5	4.2±0.6	n.s.
18:1	46.3±1.3	46.9±0.9	n.s.
20:1	1.2±0.1	1.3±0.1	n.s.
PUFA (n-6)			
18:2	9.9±0.4	10.0±0.3	n.s.
20:4	0.1±0.0	0.1±0.0	n.s.
PUFA (n-3)			
18:3	0.4±0.0	0.4±0.0	n.s.

Values expressed as % (w/w) total fatty acids (Mean±SD). § All measurements made 48 h after administration of radioactive fatty acids.

Table E.17 *Adipose tissue triacylglycerol n-6 diene content, radioactivity and specific activity of rats fed a control or cholesterol enriched diet for 30 days* §

Measurement	Control	Chol.	Significance
N-6 diene radioactivity ψ	5700±2200	5800±2100	n.s.
N-6 diene content ξ	2280±650	2450±210	n.s.
N-6 diene specific activity	2.5±0.6	2.3±0.7	n.s.

Values expressed as Mean±SD. ψ DPM/10 mg adipose tissue, ξ nmol/10 mg adipose tissue. § All measurements made 48 h after administration of radioactive fatty acids.

Table E.18 *Adipose tissue triacylglycerol [^{3}H]18:0 metabolism of rats fed a control or cholesterol enriched diet for 30 days* §

Measurement	Control	Chol.	Significance
[^{3}H]SFA	2060±540	1890±1040	n.s.
[^{3}H]MUFA	560±210	540±120	n.s.

Values expressed as DPM/10 mg adipose tissue (Mean±SD). § All measurements made 48 h after administration of radioactive fatty acids.

Appendix E

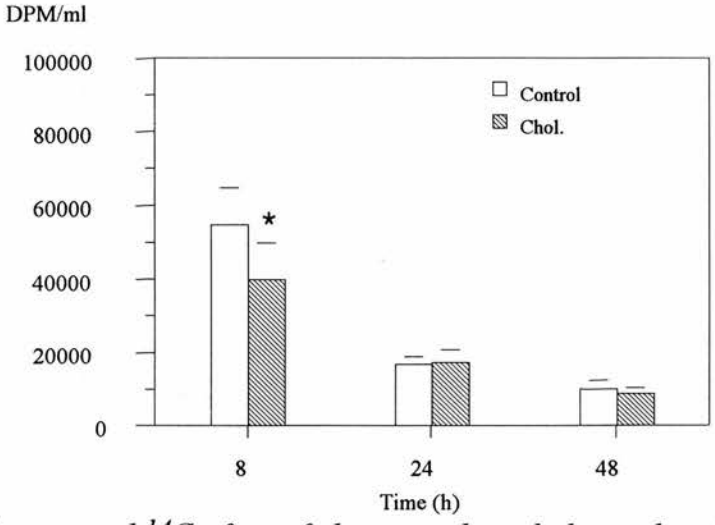

Figure E.1 *Plasma total ^{14}C of rats fed a control or cholesterol enriched diet for 56 days at 8, 24 and 48 h after administration of [1-^{14}C]18:2 n-6. * $p<0.05$ compared with control by unpaired t-test.*

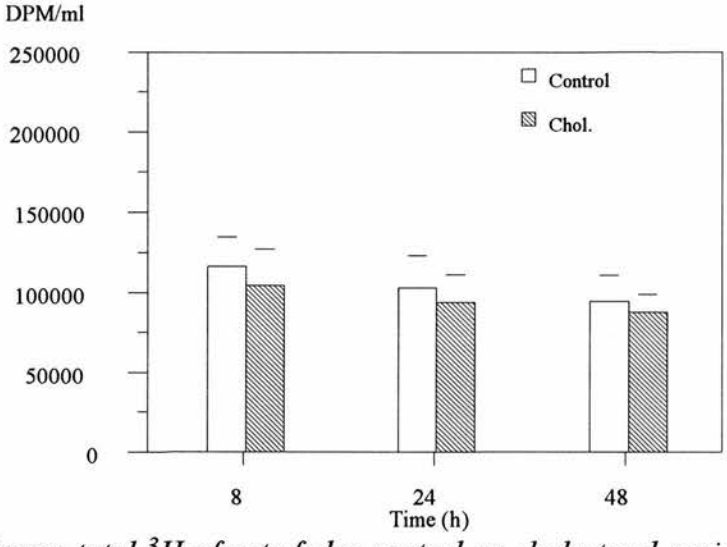

Figure E.2 *Plasma total ^{3}H of rats fed a control or cholesterol enriched diet for 56 days at 8, 24 and 48 h after administration of [11,12-^{3}H]18:0. No significant differences observed.*

Appendix E

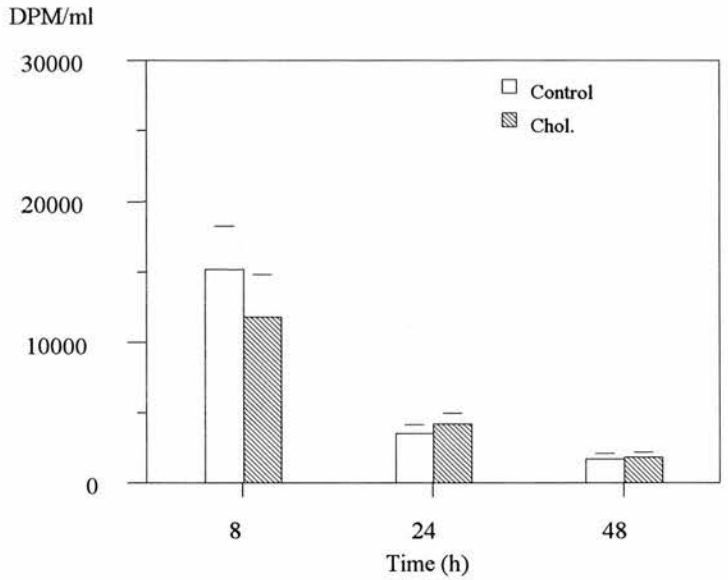

Figure E.3 *[^{14}C]labelled plasma phospholipids in rats fed a control or cholesterol enriched diet for 56 days at 8, 24 and 48 h after administration of [1-^{14}C]18:2 n-6. No significant differences observed.*

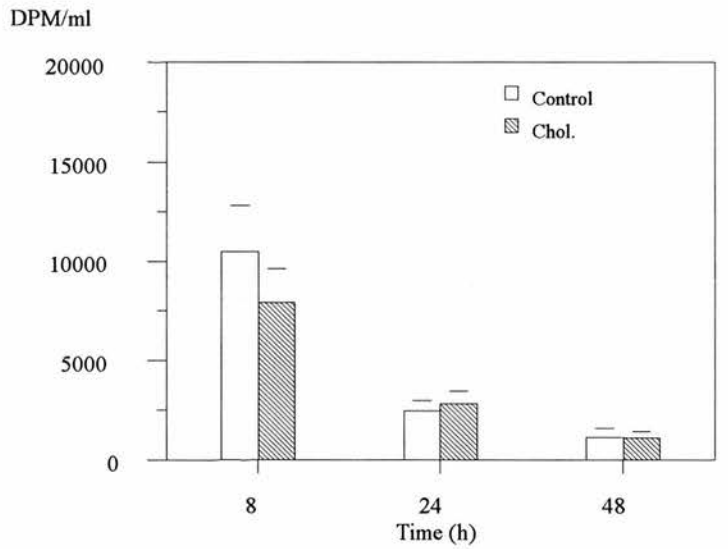

Figure E.4 *[^{3}H]labelled plasma phospholipids in rats fed a control or cholesterol enriched diet for 56 days at 8, 24 and 48 h after administration of [11,12-^{3}H]18:0. No significant differences observed.*

Appendix E

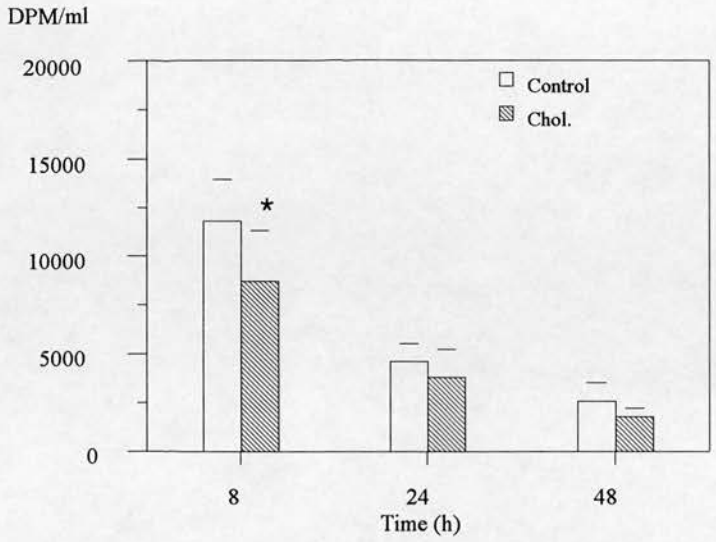

Figure E.5 *[^{14}C]labelled plasma cholesterol esters in rats fed a control or cholesterol enriched diet for 56 days at 8, 24 and 48 h after the administration of [1-^{14}C]18:2 n-6. * $p<0.05$ compared with control by unpaired t-test.*

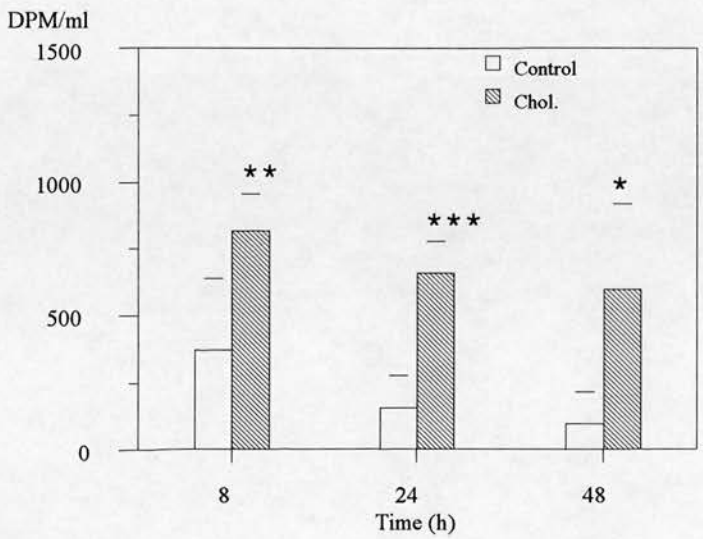

Figure E.6 *[^{3}H]labelled cholesterol esters in rats fed a control or cholesterol enriched diet for 56 days at 8, 24 and 48 h after administration of [11,12-^{3}H]18:0. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared with controls by unpaired t-test.*

Appendix F

Ingredients for the Rat and Mouse No. 1 (Modified) diet, S.D.S. Witham, Essex, U.K.

Nutrient	**Amount (%, w/w)**
Fat	2.5
Protein	14.7
Starches	45.8
Sugars	6.5
Fibre	16.5
Vitamin mix	0.5
Mineral mix	3.5
Water	10

Appendix G

Publications

Brown, J.E., Lindsay, R.M. and Riemersma, R.A. (1993) Altered delta-6-desaturase activity and fatty acid composition in liver and plasma in the spontaneously diabetic rat. In: *Abstracts of the 1st International Congress of the International Society for the Study of Fatty Acids and Lipids (ISSFAL)*, Lugano, Switzerland p. 78

Brown, J.E. and Riemersma, R.A. (1994) Effect of dietary cholesterol on delta-6-desaturase. *Scottish Medical Journal* **39**:61